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UPTAKE AND BINDING OF CARDIAC GLYCOSIDES AND THEIR  
RELATION TO CONTRACTILE EFFECT IN MYOMETRIUM

by



RAMA VENKATESA MURTHY

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Uptake and Binding of Cardiac Glycosides and Their Relation to Contractile Effect in Myometrium," submitted by Miss Rama Venkatesa Murthy in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....*E. E. Daniel*.....  
Supervisor

.....*A. M. Krishnai*.....

.....*W. B. Collins*.....

.....*S. S. Srinivasan*.....

.....*A. S. Srinivasan*.....

.....*George T. Chitt*.....  
External Examiner

Date .....*July 31, 1972*.....

.....





## ABSTRACT

Studies on the uptake of  $^3\text{H}$ -ouabain and  $^3\text{H}$ -digitoxin showed that uptake of  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M) by rabbit myometrium was  $\text{Na}^+$ , ATP and temperature dependent and was reduced by  $\text{K}^+$  and by non-radioactive ouabain or digitoxin. This suggested that  $\text{Na}^+-\text{K}^+$ -ATPase was involved in the uptake of  $^3\text{H}$ -ouabain by rabbit myometrium. Uptake of  $^3\text{H}$ -digitoxin ( $5 \times 10^{-8}$  M) by rabbit myometrium was not reduced by either  $\text{K}^+$  or non-radioactive glycosides.

In subcellular fractionation experiments, after uptake,  $^3\text{H}$ -ouabain and  $^3\text{H}$ -digitoxin ( $5 \times 10^{-8}$  M) were found to be bound mostly to  $\text{F}_1$  of rabbit myometrium. Binding of  $^3\text{H}$ -ouabain to  $\text{F}_1$  was  $\text{Na}^+$ -dependent and reduced maximally by 9.2 mM  $\text{K}^+$  to 0.2 pmoles/mg protein, and by non-radioactive digitoxin ( $10^{-5}$  M) to 0.1 pmoles/mg protein. In  $\text{K}^+$ -free media binding to  $\text{F}_1$  was maximal and amounted to 2.7 pmoles/mg protein. Binding of  $^3\text{H}$ -digitoxin was also  $\text{Na}^+$ -dependent but the total amount bound to  $\text{F}_1$  (0.68 pmoles/mg protein) was much lower than the amount of  $^3\text{H}$ -ouabain bound. Also 23 mM  $\text{K}^+$  was required to reduce maximally the binding of  $^3\text{H}$ -digitoxin to  $\text{F}_1$  to 0.22 pmoles/mg protein. Non-radioactive ouabain ( $10^{-5}$  M) reduced the binding to 0.1 pmoles/mg protein.

Studies on binding to isolated plasma membrane fraction showed that while  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M) binding was stable (only reduced 25% by 2 washes),  $\text{Mg}^{++}$ , ATP and  $\text{Na}^+$ -dependent and reduced by 5 mM  $\text{K}^+$ ,  $^3\text{H}$ -digitoxin ( $5 \times 10^{-8}$  M) binding was unstable (reduced 70% by 2 washes) and



neither  $\text{Na}^+$  nor ATP-dependent and any  $\text{K}^+$  effect was statistically insignificant.

Binding of digitoxin on exposure to  $2.5 \times 10^{-7}$  M was also not ATP or  $\text{Na}^+$ -dependent. But binding of both the concentrations of digitoxin was  $\text{Mg}^{++}$ -dependent and reduced by unlabelled digitoxin ( $10^{-5}$  M). These results suggest that  $^3\text{H}$ -ouabain binds to  $\text{E}_2 - \text{P}$  form of  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  and that digitoxin may not bind to this form at the above concentrations. As binding of  $^3\text{H}$ -digitoxin was dependent on  $\text{Mg}^{++}$ , it is possible that it binds to a conformationally altered  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  and not to the native enzyme.

Uptake of  $^3\text{H}$ -ouabain ( $10^{-6}$  M and  $10^{-4}$  M) by rat myometrium could not be shown to be dependent on  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ . This might have been due to the high non-specific uptake in the case of rat tissue which could obscure the specific uptake. Therefore, subcellular distribution and binding of  $^3\text{H}$ -ouabain ( $10^{-4}$  M and  $5 \times 10^{-7}$  M) to isolated plasma membrane fraction ( $\text{F}_1$ ) were carried out. These studies also failed to show that the binding to  $\text{F}_1$  was dependent on  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ , as this binding was neither  $\text{Na}^+$  nor ATP-dependent and neither  $\text{K}^+$  nor unlabelled ouabain ( $10^{-3}$  M) reduced this binding. These studies showed the greater lability of ouabain binding to rat compared to rabbit myometrial plasma membrane.

Studies on the action of these glycosides on contractile function of rabbit myometrium showed that both ouabain and digitoxin ( $5 \times 10^{-7}$  M) potentiated acetylcholine induced contractions within 10 minutes and this action disappeared on washing with drug free NKR within



10 minutes. The binding of  $^3\text{H}$ -ouabain ( $5 \times 10^{-7}$  M) to  $\text{F}_1$ , however, did not wash off completely in 10 minutes (only 23% of total ouabain bound was released) and also this binding was not reduced by 23 mM  $\text{K}^+$  in 10 minutes, though  $\text{K}^+$ -free media increased the binding in 10 minutes. Therefore, in the presence of 4.6 mM  $\text{K}^+$  (i.e., the  $\text{K}^+$  in NKR) no  $\text{Na}^+$ - $\text{K}^+$ -ATPase dependent binding occurred in 10 minutes, but in  $\text{K}^+$ -free, as the rate of ouabain binding is higher, binding to  $\text{Na}^+$ - $\text{K}^+$ -ATPase occurs in 10 minutes. Thus the contractile effect (carried out in NKR) occurring in 10 minutes may not be related to the binding of these drugs to  $\text{Na}^+$ - $\text{K}^+$ -ATPase.

Studies on the effect of ouabain and digitoxin on ion recovery of  $\text{Na}^+$ -rich tissues showed that  $5 \times 10^{-7}$  M ouabain or digitoxin did not inhibit ion recovery in 10 minutes. Even  $10^{-5}$  M of these glycosides did not inhibit ion recovery in 10 minutes. On 30 minutes exposure, all the concentrations (30% with  $5 \times 10^{-7}$  M and  $10^{-6}$  M and 50% with  $10^{-5}$  M) inhibited ion recovery. These results suggest that inhibition of  $\text{Na}^+$ - $\text{K}^+$ -ATPase is not the cause of potentiation of contractile effects as these two effects could be dissociated.





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## LIST OF ABBREVIATIONS

NKR	Normal Krebs Ringer
PM	Plasma membrane
SR	Sarcoplasmic Reticulum
dpm	disintegrations per minute
pNPPase	p-Nitrophenyl phosphatase
T/M	Tissue to medium ratio
S.E.	Standard Error
DNP	Dinitrophenol
IAA	Iodoacetic acid
ATP	Adenosine triphosphate
EM	Electron microscopy



## CHAPTER I

### GENERAL INTRODUCTION



## CHAPTER I

## GENERAL INTRODUCTION

Two centuries ago Withering (1785) observed the beneficial effects of digitalis in treating failing hearts, but the mechanism of its inotropic action is still undecided. Contrary to earlier belief (Bing et al, 1950, Dresdale et al, 1959), it is now well known that digitalis exerts a direct stimulatory action on the force and speed of contraction of both failing and non-failing hearts (Cotten and Stopp, 1958, Mason and Braunwald, 1963, Mason et al, 1969, Schroder et al, 1962 and Weissler et al, 1964). So its action is not limited to enlarged failing hearts, and this eliminates the possibility that it acts by the reversal of processes unique to the failing heart.

Cardiac glycosides have no significant inotropic action when contractility is depressed by metabolic inhibitors or by deficiencies (such as in vitamin B<sub>1</sub> deficiency) that decrease myocardial ATP stores (Koch Weser, 1967). Thus ATP is needed for the action of cardiac glycosides but they do not act by increasing the availability of these high energy compounds (Braunwald and Klocke, 1965).

Some workers have proposed that the release of norepinephrine from its stores in the heart may be the mechanism of the inotropic action of cardiac glycosides (Dhalla and McLain, 1964





and Tanz, 1964). This has been proved to be wrong by recent studies which showed that cardiac denervation did not alter the inotropic effect of cardiac glycosides (Morrow et al, 1963 and Spann et al, 1967). Thus the inotropic effect is direct and not mediated through nervous mechanism. (See also Moe and Han, 1969). The arrhythmia and a small part of inotropic effect may be adrenergic.

A direct action on the contractile proteins has not been shown to be the mechanism of action of cardiac glycosides. The studies, which showed an influence on physicochemical behaviour (Horvath et al, 1949) by cardiac glycosides, such as conversion of actin to F-actin (Horvath et al, 1949), or superprecipitation of cardiac myosin B and natural actomyosin (Stowring et al, 1966) were found to be due to the presence of contaminants (Jenney et al, 1967), and some of these effects had no relation with cardiotonic potency of different glycosides (Kuschinsky et al, 1952). The enzymatic activity of myosin or actomyosin was not influenced by digitalis (Katz, 1970 and Miyahara et al, 1962). Katz further reported that cardiac glycosides do not modify the affinity of "troponin" for  $\text{Ca}^{++}$ . Contractile proteins from failing hearts have been reported to be different from normal hearts (Kako and Bing, 1958), and these authors feel that cardiac glycosides act differently on the contractile proteins of failing hearts. This fails to explain, as mentioned earlier, the action of these glycosides on normal hearts. In short, as quoted by Katz, "Taken together, these studies fail to give a



consistent picture of a direct interaction between the contractile proteins and cardiac glycosides that could account for the positive inotropic action of these agents" (Katz, 1970).

Given the above findings one is led to examine the actual process of contraction and see if any of the parameters in the contractile process is affected by cardiac glycosides. Many of the recent reviews on the mechanism of cardiac glycoside action have dealt exhaustively with this particular aspect (Hecht, 1970, Koch-Weser, 1967, Lee and Klaus, 1971, Luchi and Conn, 1965, Mason, 1969 and Wilbrandt, 1966). They all agree that the insufficient knowledge concerning the contractile process has to some extent hampered the proper understanding of the inotropic effect of cardiac glycosides.

The inotropic effect of cardiac glycosides is shown to be similar to the contractile change produced by an increased heart rate, extrasystolic potentiation and increased  $\text{Ca}^{++}$  concentration in the medium (Langer, 1968 and Sonnenblick, 1967). All these procedures increased the velocity of intrinsic shortening and the intensity of the active state. Both these effects cause an increase in the force of contraction and as cited by Holland and Sabatini-Smith (1969), this is "...a finding which probably means that digitalis affects a cellular function that governs both these parameters".

The contraction process involves four proteins, two primary and two secondary proteins. The primary proteins namely actin and myosin, interact to cause contraction and the energy is



provided by ATP. Tropomyosin and troponin are contained in the thin actin filaments. Troponin in combination with tropomyosin inhibits actin and myosin interaction. Ionized  $\text{Ca}^{++}$  is required to initiate, control and maintain the interaction between actin and myosin. In its absence or in amounts less than  $10^{-7}$  M, the primary protein interaction does not occur and contraction is weak or absent (Hecht, 1970). The Troponin molecule has considerable affinity for  $\text{Ca}^{++}$ . As free  $\text{Ca}^{++}$  rises above  $10^{-7}$  M,  $\text{Ca}^{++}$  binds to an increasing number of troponin sites and by an undefined mechanism removes the troponin inhibition of bridge formation between actin and myosin. The bridge formation leads to an increase in tension development (Langer, 1971).

The excitatory and contractile events are coupled by  $\text{Ca}^{++}$  ion. The concentration of  $\text{Ca}^{++}$  rises following excitation to couple mechanical work to electrical event and  $\text{Ca}^{++}$  is removed by some "relaxing factor" to terminate the active contractile state (Weber et al, 1964).  $\text{Ca}^{++}$  is delivered to and removed from reactive sites, from storage sites which has been shown (Constantin et al, 1965) to be within the so-called sarcoplasmic reticulum (SR) system. This system forms special connections with plasma membrane facing the intracellular space at the intercalated discs and at the transverse invaginations (Page, 1966). It has been proposed (Huxley, 1959) that these connections transmit the electrical impulse from the plasma membrane to the contractile element in the skeletal muscle.



Though the SR in heart muscle (Page, 1966) and in smooth muscle (Land and Rhodin, 1964) is not very extensive, it has the same function as that of skeletal muscle (Carsten, 1964, Carsten, 1969, Inesi et al, 1964 and Simpson and Oertel, 1962). In addition to SR in the cardiac cell, sarcolemma is implied in calcium movements into and out of the cytoplasm, based on several experimental observations (Langer, 1968). It is well known that cardiac contraction is influenced by the calcium concentration in the extracellular fluid (Ringer, 1883). It has also been shown that there is an increased transsarcolemmal flux of calcium into cardiac muscle cell during excitation, (Langer and Brady, 1963 and Winegard and Shanes, 1962) and that calcium movements have been postulated to contribute to the total depolarizing membrane current (Reuter and Beeler, 1969). Thus extracellular calcium is a source of activating calcium ions. Sanborn and Langer (1970) reported that contractile dependent  $\text{Ca}^{++}$  arises from superficial sites (sarcolemmal sites) because lanthanum reduced the tension in aretrially perfused rabbit septa. But SR has been shown to be located just below sarcolemma. Therefore, the removal of  $\text{Ca}^{++}$  from sarcolemmal site by lanthanum would lead to filling of these sites with SR calcium (Solaro, 1971).

There are also reports (Kulczycky and Mainwood, 1972) which claim that SR is open to extracellular space;  $\text{Ca}^{++}$  removed is at outside of plasma membrane, consequently the loss of  $\text{Ca}^{++}$  with lanthanum could be due to its easy access to SR, which would then result in loss of tension. In







the smooth muscle it is suggested that extracellular influx of  $\text{Ca}^{++}$  activates contraction (Peachy and Porter, 1959, Somlyo and Somlyo, 1968). Somlyo and Somlyo (1968) suggested that contribution of SR and the extracellular  $\text{Ca}^{++}$  may vary in different types (eg., phasic or tonic) of smooth muscle. Uterine muscle has been shown to contract in response to strong electrical stimulus in  $\text{Ca}^{++}$ -free media (Csapo, 1971) and acetylcholine and other stimulants can cause contraction of smooth muscle in  $\text{Ca}^{++}$ -free solutions (Daniel, 1963). These results suggest the importance of intracellular  $\text{Ca}^{++}$  source in contraction.

It is generally accepted that the depolarization of surface membrane releases  $\text{Ca}^{++}$  which in turn triggers the contraction of actomyosin (Ebashi et al, 1967, Hasselbach, 1964, Winegard and Shanes, 1962). This is followed by relaxation which complete the coupling of excitation to contraction.

The passive  $\text{Na}^{+}$  entry during depolarization (excitation) is believed to be dependent on removal of  $\text{Ca}^{++}$  from membrane sites (Hecht, 1970). It is proposed by Langer (1968) that inside the muscle cell the amount of  $\text{Ca}^{++}$  on the sarcotubular membrane and sarcolemmal membranes is regulated through a competition with  $\text{Na}^{+}$  ions for a binding site. Following an increase in the frequency of heart beat, there is an increase in the intracellular  $\text{Na}^{+}$  due to  $\text{Na}^{+}$  pump lag (Langer, 1968, 1971), which releases  $\text{Ca}^{++}$  from the binding sites, and provides more  $\text{Ca}^{++}$  for increased force of contraction. Langer (1968, 1971) extends this theory to the action of



cardiac glycosides as well. The termination of active contractile state is brought about by active pumping of the  $\text{Ca}^{++}$  back to sarcoplasmic reticulum (SR) with the aid of an ATPase requiring  $\text{Mg}^{++}$ , and excess  $\text{Na}^+$  is pumped out into the extracellular space using energy obtained from a membrane ATPase-dependent on  $\text{Na}^+$  and  $\text{K}^+$  for activation. The excitation contraction coupling in smooth muscle follows the same pattern (Bohr, 1964, Daniel, 1965, Edman and Schild, 1962, Hinke, 1965).

Thus, the contractile process consists of (Lee and Klaus, 1971):

1. Propagation of action potential and spread of excitation, entrance of  $\text{Na}^+$ .
2. Flow of the transmembrane inward  $\text{Ca}^{++}$  current and release of  $\text{Ca}^{++}$  from intracellular binding site.
3. Diffusion of  $\text{Ca}^{++}$  to the contractile element and binding of  $\text{Ca}^{++}$  to troponin.
4. Interaction of ATP and actomyosin.
5. Re-uptake of  $\text{Ca}^{++}$  and its outward transport.

The effect of cardiac glycosides on diffusion of  $\text{Ca}^{++}$  to contractile elements is not known and cardiac glycosides do not seem to alter the binding of  $\text{Ca}^{++}$  to troponin (Katz, 1970). Their effect on ATP-actomyosin interaction is also not proved. An effect on action potential mechanism does not seem to be the cause of inotropic effect because the contractile force begins to increase



before there are any detectable changes in the action potential (Kassebaum, 1963 and also see Prasad and MacLeod, 1969) and the contractions initiated by immersing heart muscle in high potassium media are also augmented (Otsuka and Nanomura, 1963). They also do not have any effect on the inward  $\text{Ca}^{++}$  current at inotropic concentration (Lee and Klaus, 1971).

Inhibition of  $\text{Ca}^{++}$  uptake by cardiac sarcoplasmic vesicles or relaxing factor activity has been described by Luckenbach and Lullman (1963) by Klaus and Lee (1969) and Klaus (1967) but it was found that very high concentration of drug was required for this inhibition and there was no correlation of this effect with inotropic effect (Lee and Choi, 1966). Lee et al, (1969) have recently shown that ouabain did not influence the active  $\text{Ca}^{++}$  transport mechanism of SR but altered their  $\text{Ca}^{++}$  binding property (See Lee and Klaus, 1971). Carsten (1967) found that ouabain inhibited  $\text{Ca}^{++}$  uptake by aged SR preparations and Briggs et al (1966) showed that barbiturate inhibition of  $\text{Ca}^{++}$  uptake by SR was reversed by ouabain. Chimoskey and Gergely (1968) and Pretorius et al (1969) reported that ouabain had no effect on  $\text{Ca}^{++}$  uptake by SR. These findings are inconclusive and an effect on SR has not been established as the mechanism of action of cardiac glycosides.

There does not seem to be a significant change in the total  $\text{Ca}^{++}$  content of heart after therapeutic doses of cardiac glycosides. Digitoxin did not affect  $\text{Ca}^{45}$  efflux from perfused guinea pig hearts preloaded with  $\text{Ca}^{45}$  (Harvey and Daniel, 1952).



Lullman and Holland (1962) and Klaus and Kuschinsky (1962) found that the amount of  $\text{Ca}^{45}$  was more in cardiac glycoside treated muscle than the control muscle, but the total  $\text{Ca}^{++}$  content was unchanged or even lowered with therapeutic doses of cardiac glycosides at equilibrium, suggesting an increase in the rate of exchange of the exchangeable  $\text{Ca}^{++}$  fraction. This fraction may be similar to the exchangeable  $\text{Ca}^{++}$  fraction related to contractile effect as described by Langer (1968). Langer found that this fraction increased with reduction in  $\text{Na}^+$ , increased  $\text{Ca}^{++}$  and with increased frequency of heart rate. Holland and Sabbitini-Smith (1969) showed that ouabain increased the rate of tension decline in low  $\text{Ca}^{++}$  media and interpreted these results to imply that ouabain enhanced the release of  $\text{Ca}^{++}$  from storage sites or facilitated a passive transmembrane movement of  $\text{Ca}^{++}$ . They found that this effect depended on heart rate. Sekul and Holland (1960) found that in quiescent preparations there was no change in exchangeability of cellular  $\text{Ca}^{++}$  (See also Grossman and Furchgott, 1964). With these available results it is difficult to explain how cardiac glycosides cause contraction in quiescent heart muscle preparations (See Lee and Klaus, 1971) or in the uterine muscle preparations (Daniel, 1964 a,b).

The one effect of cardiac glycosides which is universally accepted is its specific inhibition of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  present in the plasma membrane (PM) (Glynn, 1964; Hoffman, 1966; Repke, 1961; Schatzmann, 1963 and Skou, 1965). An intimate correlation between this inhibitory action and cardiotonic potency was first shown by Repke (1961).







Besch et al (1970) and Akeris et al (1970) have shown that dog hearts perfused (in vivo) with concentration of ouabain that produced positive inotropic effect had a lower  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity than the untreated hearts; and this finding is taken as proof for the causal relationship. Lee and Klaus (1971) caution against any quantitative comparison between in vitro and in vivo effect. The main objection is most enzymes operate with a fraction of their maximal capacity in vivo and this may be one of the reasons for a failure to see any increase in intracellular  $\text{Na}^+$  with the inhibition of  $\text{Na}^+$  pump or  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, because a slight inhibition would be compensated by an increased capacity of the enzyme. On the other hand, the enzyme activity is maximal under in vitro conditions, because the experimental environment is constructed to be optimal for enzyme activity. However, the proponents of the view suggest that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  inhibition leads to an increase in intracellular sodium, whose content is low and hence much more sensitive to slight inhibition of  $\text{Na}^+$  pump and thus the increase in  $\text{Na}^+$  content may be related to inotropic effect of cardiac glycosides. (Also  $\text{Na}^+$  could be increased locally; near pm, SR and not throughout heart). Glynn (1969) proposed that if competition between  $\text{Na}^+$  and  $\text{Ca}^{++}$  at the inner surface of the membrane (and also as proposed by Langer, 1968) a rise in internal  $\text{Na}^+$  reduces the  $\text{Ca}^{++}$



efflux by displacing  $\text{Ca}^{++}$  ions and if the mobility of  $\text{Na}^+$  carrier complex is higher than  $\text{Ca}^{++}$  carrier complex an increased influx of  $\text{Ca}^{++}$  would occur. Lee et al (1970) suggested that an increase in internal  $\text{Na}^+$  caused an increase in  $\text{Ca}^{45}$  influx (Baker et al, 1969) resulting in increased contractile effect. They concluded that inhibition of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  was intimately related to inotropic effect. However, they did not find an increase in tissue  $\text{Na}^+$  content at the initial inotropic stage, even though  $\text{Ca}^{45}$  influx was increased. They proposed that there was an undetectible increase in  $\text{Na}^+$  at the inner surface of the membrane during initial stage. This was not measurable because of the small increase at localized sites. But at the same time, this increase in  $\text{Na}^+$  caused the increased  $\text{Ca}^{++}$  influx. A direct evidence for such a causal relationship is still lacking.

Besch and Schwartz (1970), on the other hand, suggest that cardiac glycosides cause a conformational change in  $\text{Na}^+-\text{K}^+-\text{ATPase}$  leading to an increased affinity for  $\text{Ca}^{++}$  at  $\text{Na}^+$  binding sites (which may not necessarily be the same as  $\text{Na}^+$  activating site of the enzyme). This results in an increase of labile  $\text{Ca}^{++}$  store at the membrane providing more  $\text{Ca}^{++}$  for contractile effect. All these suggestions need to be proved experimentally, which is no mean task. To conclude regarding an action on  $\text{Na}^+-\text{K}^+-\text{ATPase}$  in relation to the inotropic response in Glynn's words: "This explanation of the cardiotonic effect must remain only a hypothesis, though it has the advantage over rival hypotheses that it requires no new or doubtful action of cardiac glycosides".



The relationship between binding of cardiac glycosides to  $\text{Na}^+-\text{K}^+-\text{ATPase}$  and contractile effect is discussed in Chapter II.

The present study deals with the uptake and binding of two cardiac glycosides viz ouabain and digitoxin by rabbit and rat myometrium. Ouabain and digitoxin have different lipid solubilities, ouabain being less lipid soluble than digitoxin but they are approximately equipotent in their contractile effect (Cottell and Gold, 1941). Rabbit and rat have different sensitivities to cardiac glycosides, rabbit being more sensitive than rat (Akera et al, 1969; Allen and Schwartz, 1968; Daniel, 1964 a; Detweiler, 1967; Dransfield, 1966; Okita, 1967 and Repke, 1961). Cardiac glycosides have the same effect on smooth muscle as on cardiac muscle that is, to potentiate drug induced contractions at low doses and cause contracture at higher doses (Daniel, 1964 b).

This thesis attempts to answer the following questions:

1. If there is any correlation between lipid solubility of the cardiac glycosides and amount taken up by tissue and bound to specific receptors, i.e., to  $\text{Na}^+-\text{K}^+-\text{ATPase}$ .
2. If  $\text{Na}^+-\text{K}^+-\text{ATPase}$  is the receptor for contractile effect, then will these glycosides, having equal potency but different physical properties, bind to the same extent to these receptors and with similar characteristics.
3. What is the difference in binding characteristics between sensitive and insensitive species?
4. What is the correlation between the binding of ouabain



to  $\text{Na}^+ - \text{K}^+$ -ATPase to its contractile effect in the myometrium and can these two effects be dissociated?





## CHAPTER II

### LITERATURE REVIEW



## CHAPTER II

## UPTAKE AND SUBCELLULAR DISTRIBUTION OF CARDIAC GLYCOSIDES IN MYOCARDIUM

The uptake of cardiac glycosides by cardiac and other types of tissues has been studied by various workers. The results are varied and it has still not been shown as to which particular cellular structure it is bound when taken up. Harvey and Pieper in 1955 were the first to study the uptake of  $^{14}\text{C}$ -digitoxin by guinea pig hearts. They reported that the particulate fraction contained more digitoxin than the supernatant and concluded that digitoxin binds to heart tissue. Spratt and Okita in 1958 studied the binding of digitoxin to rat hearts and found that very little was bound to rat heart and the supernatant contained free digitoxin. This difference in digitoxin binding between rat and guinea pig heart has been attributed to a lower affinity of the rat heart, species difference, etc. (Dutta et al, 1968). Fisch et al (1959) who studied the ultracentrifugal distribution pattern of toxic doses of  $^3\text{H}$ -digoxin in bullfrog hearts found it to be closely associated with the microsomal fraction.

Kuschinsky et al (1968 a, 1968 c, 1967) have studied in detail the kinetic behaviour of the uptake and release of cardiac glycosides such as  $^3\text{H}$ -ouabain,  $^3\text{H}$ -digoxin and  $^3\text{H}$ -digitoxin by guinea pig atria. They found that the rate as well as the content of the uptake of these glycosides differed and only a small amount of  $^3\text{H}$ -ouabain was taken up in contrast to a very large uptake of  $^3\text{H}$ -digitoxin.



They suggested that  $^3\text{H}$ -ouabain bound only to the specific receptors related to pharmacological effect whereas most of digitoxin was bound to non-specific receptors unrelated to inotropic effect.

They found in addition that the uptake was not influenced by high  $\text{Ca}^{++}$  or low  $\text{Ca}^{++}$  and a change in the frequency of heart beats altered only the rate of uptake but not the total amount taken up at equilibrium. They also reported that unlabelled glycosides when present before the uptake of tritiated glycoside prevented the uptake but had no effect on the release of tritiated glycosides from the atria. In a later paper, Lullmann et al (1969) reported that more than 50% of  $^3\text{H}$ -ouabain uptake was not related to inotropic effect.

Dutta et al (1968 a) studied the subcellular distribution of  $^3\text{H}$ -digoxin in isolated guinea pig and rat hearts and found that guinea pig hearts accumulated more  $^3\text{H}$ -digoxin than the rat heart. But in both the cases microsomal fraction contained the highest amount of  $^3\text{H}$ -digoxin. They eluted the supernatant containing radioactive digoxin through Sephadex (G 100) and found that it was not bound to any protein. In another series of experiments they (Dutta et al, 1968 b) studied the uptake and subcellular distribution of  $^3\text{H}$ -digoxin and five other glycosides ( $^3\text{H}$ -ouabain,  $^3\text{H}$ -dihydro ouabain,  $^3\text{H}$ -digitoxin,  $^3\text{H}$ -procillaridin and  $^3\text{H}$ -convallotoxin). These studies showed that the amount of  $^3\text{H}$ -dihydro ouabain taken up was lowest and that of  $^3\text{H}$ -procillaridin was the highest when guinea pig hearts were perfused for 64 minutes followed by a 8-minute washout. Upon fractionation in all instances the highest concentration of each



glycoside was found in the microsomal fraction and again the dihydro ouabain content was the lowest and proscillaridin the highest. When the isolated sarcoplasmic reticulum fraction (SRF) of beef heart was incubated with these glycosides, it was seen that  $^3\text{H}$ -dihydro ouabain bound to the same extent as  $^3\text{H}$ -ouabain. They also found a marked difference in the potassium inhibition of glycoside binding to SRF.  $^3\text{H}$ -digitoxin and  $^3\text{H}$ -proscillaridin were least affected and binding of other glycosides were inhibited by about 60%. To explain these results they proposed a model consisting of a membrane transport system for these glycosides and two different SRF binding sites. They attributed the poor biological activity of dihydro ouabain to its poor transport and suggested that typical glycosides like ouabain, digoxin and convallotoxin bound to  $\text{K}^+$  sensitive site and digitoxin and proscillaridin mostly to  $\text{K}^+$  insensitive site in SRF. They further showed (Dutta and Marks, 1969) that the  $^3\text{H}$ -ouabain uptake and binding was  $\text{Na}^+$  dependent and Na/K ratio in the media determined the extent of binding. This led them to suggest a transport system for cardiac glycosides similar to the one for sugar transport suggested by Crane (1965) might exist in the heart membrane. To support this suggestion they showed that the uptake of cardiac glycoside was saturable and inhibited by other cardiac glycosides. Lowering of  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  had no effect on this uptake. Thus they suggested that polar glycosides are transported inside the heart cell by a carrier mechanism and that  $\text{Na}^+-\text{K}^+-\text{ATPase}$  might be involved in this transport. They do not,





however, clarify if the active  $\text{Na}^+-\text{K}^+-\text{ATPase}$  is involved in this transport.

Fricke et al (1969) and Gerber et al (1968) studied the uptake of  $^3\text{H}$ -ouabain and  $^3\text{H}$ -digitoxin by guinea pig hearts and found that the rate and the amount taken up was different for these glycosides but both the glycosides accumulated highest in the microsomal fraction. Similar result was obtained by Prindle et al (1971) using  $^3\text{H}$ -digoxin in cat heart. They also found that low  $\text{K}^+$  increased and high  $\text{K}^+$  decreased the binding to microsomal fraction. This  $\text{K}^+$  antagonism to  $^3\text{H}$ -digoxin uptake was also shown by Ebert et al (1963), Marcus et al (1969) and Morgan and Binnion (1970), for dog hearts in situ. Marcus et al (1971) reported that hypokalemia did not alter the amount of  $^3\text{H}$ -digoxin bound to heart in dogs. The reason for this result is not known. Cohn et al (1967) observed an increased uptake of  $^3\text{H}$ -digoxin by mice hearts with  $\text{K}^+$  deficiency. Similarly Harrison and Brown (1966) found that  $\text{K}^+$  depleted rats retained more digoxin  $\text{H}^3$  in hearts 24 hours after injection. Harrison and Wakim (1969) like Dutta et al (1970) showed that reduction in sodium concentration in the serum inhibited the uptake of  $^3\text{H}$ -digoxin. Harrison et al (1971) reported that hypomagnesemia had no effect on myocardial  $^3\text{H}$ -digoxin binding in dogs, while Goldman et al (1971) reported an increased uptake of  $^3\text{H}$ -digoxin by mice hearts in  $\text{Mg}^{++}$  deficiency.

Autoradiography combined with light microscopy or electron microscopy has been used by several workers to localize the exact



subcellular binding site of cardiac glycosides (Conrad and Baxter, 1964; Fozzard and Smith, 1965; Tubbs et al, 1964 and Waser, 1965). But the results are not conclusive probably because of the technical difficulties such as redistribution during histological preparation, poor resolution, etc., as cited by Lee and Klaus (1971) in their review on cardiac glycoside action.

#### UPTAKE BY OTHER TISSUES AND CELLS

Godfraind and Lesne in 1967 studied the uptake of  $^3\text{H}$ -digitoxin by guinea pig intestinal smooth muscle and found that this uptake was reduced by 100 mM  $\text{K}^+$  or by reducing the incubation temperature to  $27^\circ\text{C}$  from  $37^\circ\text{C}$ . In 1968 they reported from their uptake experiments with  $^3\text{H}$ -digitoxin and  $^3\text{H}$ -digoxin that the potency of cardiac glycoside was related to their affinity for binding sites. They concluded that polarity of the molecule was an important factor for binding and that non-polar digitoxin binds to a greater extent than polar digoxin and therefore digitoxin is more potent. The same authors in 1970 studied the uptake of  $^3\text{H}$ -ouabain,  $^3\text{H}$ -digitoxin, and  $^3\text{H}$ -digoxin by guinea pig intestinal smooth muscle. They found that the rate of the uptake process was similar for these glycosides, a result different from that for cardiac muscle (see Kuschinsky et al, 1968), but the total uptake differed according to the lipid solubility of the glycoside. They found two components in the uptake process; one a non-saturable component and the other a saturable component. The non-saturable



component was related to the polarity of the molecule and the saturable component to the pharmacological activity. They found that the uptake was reduced in the presence of another glycoside and that digitoxin was the most potent binding antagonist. However, this antagonism was not competitive in nature and the authors felt this was due to formation of hydrophilic and hydrophobic bonds with the binding site depending on the number of  $-CH_3$  and  $-OH$  groups on the molecule.

Results of Caldwell et al (1970), Forth et al (1969) and Greenberger et al (1969) differ from those of Godfraind and Lesne (1970). Forth et al (1969) studied the uptake of  $^3H$ -ouabain,  $^3H$ -digitoxin,  $^3H$ -digoxin,  $^3H$ -proscillaridin and  $^3H$ -donvallotoxin by isolated segments of the small intestine of rats and guinea pigs. They found that uptake was proportional to the concentration in the external media and that digoxin uptake was highest in both rats and guinea pigs. They did not find a saturable component in the uptake process for any of the glycosides studied. Similar results were obtained by Greenberger et al (1969) who studied the uptake of six tritiated glycosides by rat and guinea pig intestine. In addition, they found that the  $^3H$ -digitoxin uptake was not reduced by unlabelled glycosides, metabolic inhibitors or omission of glucose from the media in the case of rat. Both Forth et al (1969) and Greenberger et al (1969) found that ouabain was taken up to a greater extent in the guinea pig than digoxin while it was the reverse in the case of rat. Thus the species difference in relation to sensitivity (in terms of uptake) depends



on the glycoside used for the uptake. Caldwell et al (1969) reported that  $^3\text{H}$ -digoxin was taken up passively by the rat intestine but found that  $\text{K}^+$  (substituted for  $\text{Na}^+$  in the media) reduced the uptake. Ruiz-Torres and Ohlmeier (1970) on the other hand found that the absorption of  $^3\text{H}$ -digoxin by rat intestine was saturable at a certain dose range (0.5 mM - 1 mM). Above this concentration the absorption became non-saturable. They also found that  $\text{K}^+$  reduced the absorption of  $^3\text{H}$ -digoxin.

Kupfenberg and Schanker (1968) studied the uptake of  $^3\text{H}$ -ouabain by liver slices of the rat. Their results showed that  $^3\text{H}$ -ouabain was taken up against an apparent concentration gradient of about four-fold. The uptake was found to be saturable and reduced by metabolic inhibitors, anaerobic conditions and by other active cardiac glycosides. They did not, however, find an appreciable binding of  $^3\text{H}$ -ouabain to liver homogenate, thus they suggested that the ouabain taken up actively is free inside the liver cell and is excreted into bile. Kupfenberg in 1969 found that several naturally occurring steroids such as testosterone, progesterone and corticosterone depressed competitively the  $^3\text{H}$ -ouabain uptake by liver slices and he suggested that the steroid nucleus of cardiac glycoside was important for this uptake process.

In 1970 Landowne and Ritchie reported the binding of tritiated ouabain to mammalian non-myelinated fibres and corresponding inhibition of the electrogenic  $\text{Na}^+$  pump. By varying the ouabain concentration and the external potassium concentration they distinguished







two kinds of binding sites: "a first site specifically associated with pumping and whose ability to bind ouabain was dependent on the external presence of potassium and a second site not associated with pumping and unaffected by external potassium". The second site was non-specific and non-saturable. They further tried to distinguish these two binding sites by adding 50 mM Cs to the incubation media. But  $\text{Cs}^+$  had no effect on either type of binding which is in disagreement with the results of Hoffman (1969) who reported that Cs reduces only the non-specific binding and the pump associated binding sites in red cells were not affected by  $\text{Cs}^+$ . He found that when red cells were incubated in the presence of Cs and  $^3\text{H}$ -ouabain, the  $\text{Na}^+$  pump was inhibited to the same extent as in the absence of  $\text{Cs}^+$  but the binding of  $^3\text{H}$ -ouabain was reduced to 50% of the control level. Hoffman found that  $^3\text{H}$ -ouabain uptake by human red cell ghosts was increased in the presence of ATP and reduced by unlabelled ouabain. It was also dependent on  $\text{Mg}^{++}$  but was unaffected by changes in  $\text{Na}^+$  or  $\text{Ca}^{++}$  concentrations in the media. The uptake was also supported by other nucleotides such as ITP, CTP, ADP, UTP and GTP. Hoffman prefers to interpret these results on the basis of a change in membrane conformation. He feels that phosphorylation is not a necessary step for binding of  $^3\text{H}$ -ouabain but a change in membrane conformation produced by various nucleotides leads to ouabain binding. This changed conformation is stable once the glycoside is bound and the  $\text{Na}^+$  pump is thus inhibited. (But see a recent report by Tobin et al 1972). This binding is antagonized by external  $\text{K}^+$ .



The results of Baker and Willis (1969) are more informative. They used Hela cells which are more active than red cells in pumping out  $\text{Na}^+$  and compared the extent of this binding with red cells and rabbit and guinea pig kidney cells and slices. They gave seven reasons to show that the glycoside binding to tissue culture cells measures the pumping sites. They are:

1. At low glycoside concentrations the final level of binding was independent of concentration and the rate constants for binding and debinding were consistent with the measured inhibitory constant.
2. The binding was temperature dependent.
3. Binding was reduced by raising  $\text{K}^+$  concentration.
4. Binding was reduced in  $\text{Na}^+$ -free media and by metabolic inhibitors.
5. The binding was associated with cell surface and was not lost on lysis of the cells. The binding to isolated membrane was similar to the binding on intact cells.
6. The bound glycoside was released by warming to  $70^\circ$ , 50% TCA treatment, 6 M urea extraction or ethanol extraction. The ease of release argues against incorporation of the glycoside molecule into some macromolecular constituent of the cell.
7. The uptake was reduced by cold ouabain but the total number of molecules bound per cell was increased possibly due to non-specific uptake.



It is pertinent to mention here that many of these were the guidelines used to distinguish the specific binding, i.e., to  $\text{Na}^+-\text{K}^+-\text{ATPase}$  from the non-specific binding of cardiac glycosides in the present work.

#### BINDING OF CARDIAC GLYCOSIDES TO $\text{Na}^+-\text{K}^+-\text{ATPase}$ PREPARATIONS

Matsui and Schwartz in 1967 studied the binding of digoxin  $\text{H}^3$  to a  $\text{Na}^+-\text{K}^+-\text{ATPase}$  preparation from calf cardiac tissue. They found that the binding required ATP and  $\text{Mg}^{++}$  and was stimulated by  $\text{Na}^+$  and depressed by  $\text{K}^+$ . Active cardiac glycosides significantly reduced the binding while inactive ones (hexahydroscillaridin) had no effect. Noncardiotonic steroids were also without any effect. They also found that acetylphosphate substituted for ATP, promoted binding and  $\text{Na}^+$  had no effect on this binding but  $\text{K}^+$  still reduced it. They explained these results by postulating formation of a phosphorylated enzyme intermediate in the presence of  $\text{Na}^+$  and ATP or acetylphosphate and that digoxin bound to the phosphorylated enzyme intermediate. Later these authors (Schwartz et al, 1968) found that digoxin could bind to the dephospho form of enzyme as well under certain conditions. These results led them to propose that "cardiac glycoside is an allosteric inhibitor effecting a stabilization of some intermediary form of the enzyme". Albers et al in 1968 studied the interaction of ouabain and other cardiac glycosides with  $\text{Na}^+-\text{K}^+-\text{ATPase}$  preparation from Electrophorus electric organ. These



results were similar to those of Schwartz et al (1968) in some respects. In the presence of nucleotides,  $\text{Na}^+$  and  $\text{Mg}^{++}$  stimulated the rate of binding but  $\text{K}^+$  reduced it. In the presence of orthophosphate  $\text{Mg}^{++}$  increased,  $\text{K}^+$  reduced and  $\text{Na}^+$  markedly decreased the interaction. The amount of steroid bound paralleled the inhibition of enzyme activity. They found that the enzyme was inhibited in the presence of either ATP or ADP and thus whether phosphorylated or not. (Tobin and Sen, 1970 attribute this to the presence of adenylate kinase in the enzyme preparation). This prompted them to refute Repke and Portius' (1966) suggestion that the  $\alpha$ - $\beta$  unsaturated carbonyl of the lactone ring forms a hydrogen bond with the phosphorylated enzyme. The sensitivity of ouabain binding to methanol extraction, heat and acid treatment suggested that the interaction was lipophilic and that lipoprotein or nonpolar region of a peptide chain might be involved in the interaction. The binding of ouabain was not reversed when washed with cold water. This led them to suggest that the binding was irreversible. Albers et al (1968) also found that the phosphorylation of ouabain treated enzyme in the presence of orthophosphate was comparable to the maximal phosphorylation of the native enzyme with ATP. They assume that these phosphorylation sites are identical. With these evidences they suggested that ouabain reduces the free energy difference between phosphorylated and non-phosphorylated forms of enzyme. They attribute the action of ouabain to its action on the conformation of enzyme: "...which removes constraint on the structure of the enzyme which is normally a consequence of







phosphorylation". Post and his associates (1969) found that the phosphorylation sites of native enzyme with ATP and ouabain treated enzyme with orthophosphate were identical by studying the electrophoretic pattern of peptic digested fragments of the two phosphorylated enzymes. The electrophoretic mobility was similar for the two enzyme fragments.

The irreversible interaction of cardiotonic steroids with  $\text{Na}^+ - \text{K}^+$ -ATPase from beef brain was shown by Yoda and Hokin (1970), which concurs with the results of Albers et al (1968). Yoda and Hokin (1970) suggested that the sugar in glycosidic linkage with the 3-position of the steroid nucleus was important in irreversible interaction because the inhibition of enzyme by aglycones of these compounds was easily reversible. These studies are not conclusive because they did not measure the amount of glycoside bound but only the inhibition of enzyme. The results of Tobin and Sen (1970) are more explicit in resolving the question of reversibility of ouabain binding to  $\text{Na}^+ - \text{K}^+$ -ATPase. They studied the effect of temperature on ouabain-enzyme complex and found that the complex was stable at  $0^\circ\text{C}$  but unstable at higher temperatures. They also studied the effect of different ions on ouabain binding to  $\text{Na}^+ - \text{K}^+$ -ATPase from guinea pig kidney and found that native enzyme bound very little ouabain below a concentration of  $10^{-6}$  M. The specific binding occurred in two ways. One in the presence of  $\text{Mg}^{++}$  or  $\text{Mg}^{++}$  and  $\text{Pi}$  which was saturated at  $10 \mu\text{M}$  ouabain and was reduced by  $\text{Na}^+$  and EDTA.  $\text{K}^+$  at low concentrations



stimulated this binding and inhibited it at high concentrations. The second type of specific binding occurred in the presence of 200 mM  $\text{Na}^+$  and required  $\text{Mg}^{++}$  and ATP.  $\text{SrCl}_2$  or ADP did not support binding in the presence of  $\text{Na}^+$  (in contrast to the results of Schwartz et al and Albers et al). They suggested that there was one specific binding site for ouabain and ouabain binding in the presence of  $\text{Na}^+$  depends on phosphorylation of the enzyme. The binding in the presence of  $\text{Mg}^{++}$  and  $\text{Pi}$  is to the conformationally altered but not phosphorylated enzyme. This enzyme-ouabain complex, according to the authors, is similar to the  $\text{K}^+$ -sensitive phosphorylated enzyme because both have a low affinity for  $\text{Na}^+$ . They also have calculated the entropy change for the ouabain-enzyme interaction which was about 123 e.u. This high value suggested a large conformational change associated with the interaction.

There have been many more papers recently on the binding of ouabain to ATPase. It is impossible not to have overlooked a few of them. But I shall try to discuss the ones which I think are relevant to my work.

Barnett (1970) and Hansen (1971) studied the binding of  $^3\text{H}$ -ouabain to lamb brain ATPase and ox brain ATPase respectively. Barnett's results are similar to the ones already mentioned, i.e., the rate of binding was reduced by  $\text{K}^+$  and the binding and inhibition of ATPase were related. However, they found that  $\text{K}^+$  reduced only the rate of binding but not the total amount bound. Hansen found a



similar antagonistic action of  $K^+$  on ouabain binding. He also found that EDTA or unlabelled ouabain reduced this binding, and that the binding was reversible. On the basis of these results, Hansen suggested a model based on equilibrium situation requiring a forward and backward reaction. Using the model he calculated  $EG_{max}$  (Maximum Ouabain bound) which approximated to the maximum binding measured. From this he calculated the  $K_s$  which was closely related to  $K_I$  for inhibition. Apart from these kinetic studies he found that the binding of ouabain increased proportionately with the specific activity of the enzyme preparation. Thus deoxycholate treatment which increased the specific activity of the enzyme increased the binding of ouabain as well. This differs from the results of Ellory and Smith (1969) who reported that deoxycholate-induced changes in  $Na^+-K^+-ATPase$  (from gold fish intestine) were not accompanied by parallel changes in digoxin binding. (Digoxin is a polar glycoside like ouabain).

Akera and Brody (1971) and Allen et al (1971 a) have studied the effect of  $K^+$  on the formation and dissociation of ouabain- $Na^+-K^+-ATPase$  complex. Akera and Brody found that the dissociation process was slower than the binding process. They found that in the presence of 1.0 mM KCl 50% of the maximal binding occurred in 3 minutes whereas 50% release of ouabain took more than 30 minutes. They state that "...the mechanism of antagonism between KCl and ouabain seems to be the inhibition of the ouabain-enzyme

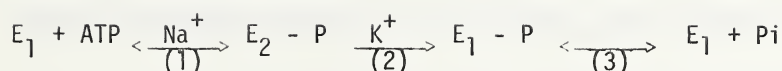


complex rather than the shifting of the equilibrium", because the inhibitor-enzyme reaction does not reach an equilibrium during the enzyme assay under conditions employed by most workers. The evidence for this non-equilibrium type of interaction was that KCl failed to antagonize ouabain action once the enzyme was inhibited by ouabain. In the absence of KCl, ouabain dissociated from enzyme during incubation at 37°C in the presence of 50 mM Tris HCl at pH 7.5, with a concomittant reversal of the enzyme inhibition. At low temperatures this complex was stable.  $K^+$  stabilized the complex once formed at concentration ranging from 0.2 to 80 mM. This effect was shown, not to be due to a change in ionic strength because  $Na^+$  or  $Li^+$  had very little effect on this dissociation. Thus KCl inhibited both the formation and the dissociation of ouabain-enzyme complex. The results are explained on the basis of an assumption that a particular allosteric configuration of enzyme favours ouabain binding and KCl alters this configuration which is unfavourable for ouabain binding. Another finding was that the complex formed in the presence of  $Mg^{++}$  and  $P_i$  decayed at a slower rate and was not affected by  $K^+$ . They thus concluded that the complex formed in the presence of  $Na^+$ ,  $Mg^{++}$  and ATP was different from the one formed in the presence of  $Mg^{++}$  and  $P_i$ , a finding different from that of Albers et al and Post et al. Akera and Brody have suggested the following scheme for ouabain enzyme interaction.

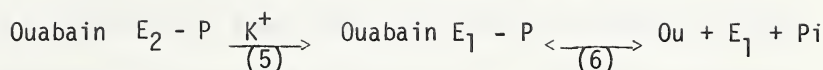








Ouabain (4)



Ou-E<sub>2</sub>-P is the unstable complex which in the presence of K<sup>+</sup> is converted to Ou-E<sub>1</sub>-P the stable form. This form is equivalent to the complex formed in the presence of Mg<sup>++</sup> and Pi. Qualitatively similar results were obtained by Allen et al (1971 a) using calf brain Na<sup>+</sup>-K<sup>+</sup>-ATPase. These results are in disagreement with Tobin and Sen's results (1970) in that they showed that Ou-enzyme complex formed in the presence of MgCl<sub>2</sub> and H<sub>3</sub>PO<sub>4</sub> at 37°C was unstable at 37°C and dissociated completely within 15 minutes. The reasons for these differences are not known. Recently Allen et al, (1971 b) found that enzyme from rabbit heart behaved differently from the beef heart enzyme. They found that Ou-E-complex formed in the presence of Mg and Pi was unstable in the case of rabbit as compared to the beef heart enzyme-ouabain complex. These results caution one against the comparison of enzyme preparation from one species to the other and the hazards in extrapolation of the results in one species or even tissue to the other (e.g., rat brain enzyme is very sensitive to ouabain inhibition while enzyme from muscle is relatively insensitive).



CARDIAC GLYCOSIDE BINDING TO  $\text{Na}^+\text{-K}^+\text{-ATPase}$  AND INOTROPIC EFFECT

Repke in 1965 studied the inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  by various glycosides and found that the inhibitory potency of each cardiac glycoside studied was equivalent to its cardiotonic potency. He then proposed that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  was the receptor for cardiotonic action. He also found that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  from rat heart was less sensitive than from that of guinea pig heart to cardiac glycosides; this would be expected on his hypothesis since rat heart was less sensitive to cardiotonic action. These results spurred the interest of pharmacologists and several attempts have been made since then to prove that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is the receptor for cardiotonic action. Schwartz and his associates, after showing that  $^3\text{H}$ -digoxin binds to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (Matsui and Schwartz, 1967), showed that the conditions required for binding were also required for the inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (1969). They then tried to correlate the inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  to cardiotonic action (Besch et al, 1970). They perfused dog heart in situ with digoxin until they got an increased force of contraction and then isolated the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  from heart. They found that the enzyme from drug perfused hearts was inhibited and in another study Allen et al (1970) showed that such an inhibited enzyme (from the ouabain perfused heart) bound less  $^3\text{H}$ -ouabain than the uninhibited enzyme. This they suggested was due to ouabain bound (during perfusion) to the inhibited enzyme. Thus these workers showed that the inhibition of enzyme was associated with binding of digoxin



and that this inhibition was correlated with the contractible effect. Similar results were obtained by Akera and Brody (1971).

Prindle et al (1970) tried to correlate inotropic effect with binding of digoxin to microsomal fraction (which is rich in  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ ). They compared the digoxin content with increase in contractile effect only at 45 minutes for low  $\text{K}^+$ , at 45 and 90 minutes for normal  $\text{K}^+$  and 45, 90 and 150 minutes for high  $\text{K}^+$  media. They found a correlation between digoxin content of the tissue in these media containing different amounts of  $\text{K}^+$  to the tension developed in cat heart. However, at equilibrium all the tissues had similar digoxin content and same active tension. This led them to propose that  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  might be the receptor for cardiotonic action because  $\text{K}^+$  has been shown to reduce the rate of binding of digoxin to  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  (Schwartz et al, 1968) but not the final amount bound. These workers, however, did not measure the digoxin content when the active tension was rising (i.e., before 45 minutes), also they seem to overlook their results which show that even though digoxin content was lower for tissues in normal  $\text{K}^+$  media and high  $\text{K}^+$  media at 90 and 150 minutes than the digoxin content at 45 minutes for tissues in low  $\text{K}^+$  media, the action of digoxin on active tension and rate of tension development were actually higher for the tissues in normal  $\text{K}^+$  and high  $\text{K}^+$  media than for the tissues in low  $\text{K}^+$  media. They have not explained the reason for these findings.

Lullmann et al (1969) correlated the decline of positive inotropic action and the loss of cardiac glycosides from isolated



guinea pig atria during washout. They found that positive inotropic effects of ouabain, digoxin and digitoxin had disappeared within 20 to 30 minutes, but the tissue concentration of these glycosides declined much more slowly. They concluded that the rate at which the glycosides diffuse out of the extracellular space probably governs the decrease in pharmacological effect. Lullman and van Zweiten (1969) studied the kinetic aspects of the inhibition of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  from red cell ghosts by ouabain and found that the ouabain-enzyme complex dissociated rapidly upon dilution of the incubating media because the enzyme was no longer inhibited upon dilution. They suggested that there was a certain parallelism between the reversibility of enzyme inhibition and the disappearance of contractile effect during washout. However, an extrapolation of red cell ghosts to heart tissue may not be justified.

The results of Fricke and Klaus (1971 a) make this picture even more confusing. They studied the reversibility of cardiotonic action of digoxin, K-strophanthidin and strophanthidin 3-bromoacetate (SBA) and found that digitoxin had the slowest rate of decline ( $t_{1/2} = 30$  minutes) and K-strophanthidin the most rapid ( $t_{1/2} = 3.5$  minutes) and SBA had an intermediate position with 70% of the effect at a  $t_{1/2}$  of 30 minutes. They suggested that SBA had two receptor sites with different affinities, one similar to that of digitoxin and one to that of K-strophanthidin (which means digitoxin and K-strophanthidin are acting on different receptors). In another series of experiments







(Fricke and Klaus, 1971 b) they studied the effect of these glycosides on microsomal ATPase of guinea pig hearts. They found that when the enzyme was treated with glycosides in the absence of  $Mg^{++}$ ,  $Na^+$  or ATP enzyme was irreversibly inhibited by about 14% with digitoxin, 10% with K-strophanthin and 33% with SBA. When the enzyme was treated in the presence of  $Mg^{++}$ , ATP and  $Na^+$ , the inhibition was 90% with all these glycosides and was fully reversible. They thus concluded that SBA inhibited irreversibly only the non-phosphorylated enzyme and therefore complete reversibility of pharmacological effect of SBA is possible if it inhibits the phosphorylated enzyme. They suggest that the phosphorylated enzyme may be predominant in the intact heart cell. This would thus explain the reversibility of SBA action on contractility. (But see Hokin, 1969, who reported a 90% irreversible inhibition by SBA). Fricke and Klaus (1971) also studied the effect of  $K^+$  on inotropic effect and enzyme inhibition caused by these glycosides. They found that only the inotropic effects of digitoxin and K-strophanthin were altered by changing  $K^+$  concentration from 5 mM to 16 mM. SBA inhibition was reversed only with 50 mM  $K^+$  and SBA was less potent at 5 mM and more potent at 16 mM than K-strophanthin in inhibiting ATPase but the potency for inotropic action was the same for both the glycosides. They thus caution against comparing the pharmacological effects carried out at 5 mM with the enzyme effect at so called optimal concentration (i.e., at 20 mM  $K^+$ ). From these results they suggested that inotropic effect of cardiac glycosides was related to  $Na^+-K^+-ATPase$ . They could not, however, explain the results with SBA, i.e., the 30%



irreversible inhibition under certain conditions and the effect of  $K^+$  on contractility.

The results of Thomas et al (1970) are in disagreement with the above results. They tested the positive inotropic effect of SBA and strophanthidinol, 19- $^3H$ -3-bromoacetate ( $^3H$ -Sol-Ba) and also their uptake by guinea pig and rabbit hearts. Both the drugs were found to have a rapid onset of action reaching maximal inotropic effect in 20 minutes. The effect disappeared on washing with drug-free buffer with a  $t_{1/2}$  of 24 minutes to SBA and Sol-Ba. When the amount of drug remaining bound was determined for  $^3H$ -Sol-Ba after 120 minutes wash, the amount bound was approximately six times higher than the concentration of drug in the media during exposure to  $^3H$ -Sol-Ba. Thus they suggested that  $Na^+-K^+$ -ATPase may not be responsible for positive inotropic action, because it has been shown by Hokin et al (1966) and also Okita et al (1969) that SBA and Sol-Ba are irreversible inhibitors of ATPase but yet the positive inotropic action is reversible. In another report (Roth-Schechter et al, 1970) where they studied the inhibition of  $Na^+-K^+$ -ATPase after perfusing the rabbit atria for three hours with SBA and then preparing the enzyme using the conventional method (i.e., extraction with 1 M Li Br), it was found that the ATPase was not inhibited. This lack of inhibition by SBA was attributed to dissociation of SBA and enzyme complex during preparation of the enzyme, because they found that 90% of SBA inhibition could be reversed by exposing it to 1 M Li Br. Thus they were unable to show if the tissue binding was



related to  $\text{Na}^+ - \text{K}^+$ -ATPase inhibition. They also mentioned that SBA and Sol-Ba being alkyl derivatives are able to alkylate non-specifically with all types of proteins and therefore, the non-specific binding of the alkyl derivatives would be much higher than their non-alkylated parent compounds. They feel that this non-specific binding is irreversible whereas the binding to pharmacological receptors is reversible. Thus the use of alkylated derivatives has a disadvantage of high non-specific binding unrelated to both inotropic effect and also  $\text{Na}^+ - \text{K}^+$ -ATPase (see Hokin, 1969).

Fujino et al (1971) tried to locate the binding site of ouabain by isolating the subcellular-fractions of the hearts at the peak of inotropic effect and found that microsomal fraction had the highest amount of ouabain  $\text{H}^3$ . They also measured the  $\text{Na}^+ - \text{K}^+$ -ATPase-activity of a preparation prepared according to Akera et al (1970) from these hearts and found that  $\text{Na}^+ - \text{K}^+$ -ATPase-activity was not inhibited, a finding different from that of Akera et al (1970) and Besch et al (1970). In another series of experiments, Fujino et al (1971) incubated cell membrane and sarcoplasmic reticulum preparations from heart and skeletal muscle and found that sarcoplasmic fraction bound more ouabain than cell membrane. Unfortunately, they do not mention the conditions used for incubation (such as the presence of or absence of ATP,  $\text{Mg}^{++}$  and  $\text{Na}^+$ ).

Pra et al (1971) studied the kinetics of ouabain uptake in frog heart and reported that activation of transport ATPase and



ouabain binding in microsomal fraction could be correlated. They did not treat the tissues in similar ways when studying the two parameters, thus the result is not very convincing.

All these results suggest two views on cardiac glycoside action, namely inhibition of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  as the cause and the other inhibition of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  not related to contractile effect. It seemed, therefore, worthwhile to study the relationship of binding of cardiac glycoside to plasma membrane to its contractile effect in myometrium. Daniel (1964 a, b) has shown that cardiac glycosides have fundamentally the same action on smooth muscle as on cardiac muscle. Cardiac glycosides potentiate the drug induced contractions at lower doses and cause contractures at higher doses in myometrium, and also rat myometrium like rat heart is less sensitive than rabbit myometrium with respect to contractility and also ion movements.

As mentioned earlier in the introduction, uptake and binding to plasma membrane and contractile effect of ouabain and digitoxin were studied.







### CHAPTER III

#### UPTAKE OF $^3\text{H}$ -OUABAIN AND $^3\text{H}$ -DIGITOXIN BY RABBIT AND RAT MYOMETRIUM



## CHAPTER III

UPTAKE OF  $^3\text{H}$ -OUABAIN AND  $^3\text{H}$ -DIGITOXIN BY RABBIT AND RAT MYOMETRIUM  
(Murthy et al, 1972)

## METHODS AND MATERIALS

## Tissue Preparation

Female rabbits (New Zealand White strain) weighing 3 - 4 pounds or pregnant rats (200 - 250 grams, Wistar strain) were used in all the experiments. Rabbits were injected subcutaneously with 200  $\mu\text{g}$  of oestrogen for 5 to 7 days. After the animals had been killed with a blow on the head or by injection of air into the marginal vein of the rabbit's ear, their uterine horns were removed and dissected free from surrounding tissue. The endometrium and circular muscle were then removed by stripping off, and the myometrial pieces were transferred immediately to a Krebs Ringer medium at  $37^\circ\text{C}$  bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  mixture. Tissue pieces weighed between 60 - 120 mg. Tissues were weighed before and after the incubation in various media.

## Measurement of Cardiac Glycoside Uptake

Tissues after equilibration in the appropriate media for 45 - 60 minutes, were transferred to media containing tritiated



glycosides in the concentration of  $5 \times 10^{-8}$  M for rabbits and  $10^{-6}$  M for rats. Tissues were incubated for 1 hour or for the length of time indicated in the text and at the end of incubation period they were removed and rinsed quickly in cold medium blotted on wet filter paper and transferred to weighed counting vials. The weight of the tissues ranged from 60 - 120 mg. After taking the tissue weight, the tissues were solubilized using 0.5 ml/100 mg of tissue of NCS solubilizer. When the tissues were dissolved, 10 ml of Bray's scintillator (1960) solution was added and the  $^3\text{H}$  content measured. Aliquots of incubation media were also counted for  $^3\text{H}$  contents in a Picker Nuclear Liquid scintillation counter (Liquimat Model 110). When metabolic inhibitors or choline or lithium chloride were in the media, the tissues were preincubated in these media for one hour before the uptake of cardiac glycoside was studied. All the vials were counted for 10 minutes and each was corrected for background counts and quenching. The quench correction was made using the "Channels ratio" method. Quenched  $^3\text{H}$  standards were counted on two channels one of which covered the entire tritium spectrum (A) whereas the others covered approximately one-third of the total spectrum (B). Since the DPM in the quenched standards were known, the counting efficiency  $\left( \frac{\text{CPM}}{\text{DPM}} \times 100 \right)$  could be correlated with the channels ratio, i.e.,  $\frac{\text{CPM in B}}{\text{CPM in A}}$  and a calibration curve constructed. The experimental samples were counted using the



same channels and after subtraction of the background activity the channels ratio was calculated and the efficiencies were read from the graph. When DNP was present in the medium, correction for colour quenching was made using the "internal standard" method. The uptake was expressed as slice to medium ratio (S/M ml/g), that is,

$$\frac{\text{DPM/g of tissue wet weight}}{\text{DPM/ml of medium}}$$

these calculations. All the samples were cooled to reduce background counts due to chemiluminescence. Measurement of extracellular space was similarly made using  $^3\text{H}$ -inulin (Taylor et al, 1970).

#### Efflux of Tritiated Cardiac Glycosides from Tissue

Tissues after incubation in the presence of  $^3\text{H}$ -ouabain or  $^3\text{H}$ -digitoxin were transferred to non-radioactive media and incubated for measured lengths of time. The efflux media were changed every 10 minutes.

#### Determination of Ion Contents

Uterine pieces were used for this experiment. Uterine pieces from rabbit or rat were incubated in Normal Krebs Ringer (NKR) in the presence of  $5 \times 10^{-8}$  M for rabbits or  $10^{-6}$  M for rats of non-radioactive glycosides for 1, 2 or 3 hours, and  $\text{Na}^+$  and  $\text{K}^+$  content of these tissues were measured. The tissues after incubation were transferred to preweighed tubes and the wet weights obtained. To





each tube 0.2 ml each of 35%  $\text{H}_2\text{O}_2$  and  $\text{HNO}_3$  solution were added and samples were digested on a sand bath kept at  $200^\circ\text{C}$ . Further lots of  $\text{H}_2\text{O}_2$  and  $\text{HNO}_3$  were added till a whitish residue was obtained. In each instance blank test tubes were processed at the same time to correct for any  $\text{Na}^+$  that may have been leached out of the glass by the acid. The residues obtained were dissolved in required amount of deionized water. The ion contents ( $\text{Na}^+$  and  $\text{K}^+$ ) were determined by flame photometry using an EEL Flame Photometer. Standard solutions of  $\text{NaCl}$  and  $\text{KCl}$  were used to construct a standard curve for each determination. Values obtained from the blanks were subtracted from the sample readings and the ionic contents read off from the standard curve. The ionic contents were expressed in terms of wet weight of tissue.  $\text{Na}^+$  and  $\text{K}^+$  contents were also measured in myometrial pieces incubated in the presence of choline or lithium chloride media.

#### SOLUTIONS, CHEMICALS AND DRUGS

All the solutions were made using double distilled deionized water. The Normal Krebs Ringer (NKR) had the following composition in mM.  $\text{NaCl}$  116,  $\text{NaHCO}_3$  22,  $\text{NaH}_2\text{PO}_4$  1.2,  $\text{KCl}$  4.6,  $\text{CaCl}_2$  1.5,  $\text{MgSO}_4$  1.2, Glucose 50.  $\text{K}^+$ -free Krebs was prepared by omitting  $\text{K}^+$  in the Krebs. Choline Krebs and Lithium Krebs were prepared by replacing  $\text{NaCl}$  with equimolar quantities of choline or lithium chloride and the pH adjusted to 7.3 with Tris. Cardiac glycosides and metabolic



inhibitors were weighed out and added directly to the Krebs solution to get the required concentration. All solutions were equilibrated with either 95% O<sub>2</sub>/5% CO<sub>2</sub> or pure O<sub>2</sub> in the case of Na<sup>+</sup>-free media. The pH of all solutions were between 7.3 - 7.5.

The chemicals and drugs used in the present study and their sources are given below.

The drugs used were ouabain (Nutritional Biochem. Corp.) and digitoxin (Sigma).

The metabolic inhibitors used were 2-4-Dinitrophenol (Fisher Chemicals) and Iodoacetic acid (Eastman Organic Chemicals).

Chlorides of Lithium and Choline were obtained from Fisher Chemicals and Trizma base from Sigma.

Radioactive substances used were <sup>3</sup>H-ouabain, <sup>3</sup>H-digitoxin and <sup>3</sup>H-inulin all from New England Nuclear Corporation, Boston, Mass. Chemical purity of <sup>3</sup>H-ouabain and <sup>3</sup>H-digitoxin was checked by thin layer chromatography (Dutta et al, 1968 and Godfraind and Lesne, 1970).

### Statistical Analysis

The variability of samples is expressed as mean  $\pm$  standard error. The significances of differences between paired samples were determined using student's t-test for paired data. Where multiple comparisons were made, Sheffe's test was used (Edwards, 1968) using a APL program which was prepared by Dr. D. A. Cook of this Department.



## RESULTS

Effect of  $5 \times 10^{-8}$  M ouabain on rabbit and  $10^{-6}$  M ouabain on rat uterine tissue  $\text{Na}^+$  and  $\text{K}^+$  content: Table 1 shows the effect of ouabain over a period of 3 hours on the  $\text{Na}^+$  and  $\text{K}^+$  content expressed as meq/g wet weight of rabbit and rat uterine tissue. There was no difference in either  $\text{Na}^+$  or  $\text{K}^+$  content of ouabain treated tissue from the control tissue over a period of 3 hours. This is the longest time of incubation used in most of the experiments in this study.

#### UPTAKE CHARACTERISTICS OF $^3\text{H}$ -OUABAIN IN RABBIT MYOMETRIUM

Rate of uptake of  $^3\text{H}$ -ouabain: Figure 1 shows the uptake of  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M) in NKR by myometrium over a period of 5 to 90 minutes. An equilibrium was reached within 60 minutes. Therefore, this period of incubation has been used throughout. The tissue to medium ratio (T/M) for  $^3\text{H}$ -ouabain was only slightly higher than the inulin space which was  $0.45 \pm 0.03$  ml/g in myometrium. T/M for ouabain was  $0.60 \pm 0.06$  ml/g at equilibrium.

Rate of uptake in  $\text{K}^+$ -free Ringer: We expected binding\* of

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\* Binding of cardiac glycosides refer to their combination with cellular components; operationally, it will be defined as the portion of uptake inhibited by a high concentration of unlabelled glycoside. For reasons that will become apparent the portion of uptake inhibited by elevated potassium concentrations will be referred to as glycoside bound to transport ATPase.



TABLE 1

Effect of ouabain on ion movements in rabbit and rat uterii

Treatment <sup>a</sup>	Rabbit <sup>b</sup>		Rat <sup>c</sup>	
	Na <sup>d</sup>	K <sup>d</sup>	Na <sup>d</sup>	K <sup>d</sup>
Control 1 hr.	93 $\pm$ 6	59 $\pm$ 5	94 $\pm$ 10	68 $\pm$ 7
Ouabain 1 hr.	95 $\pm$ 11	58 $\pm$ 6	93 $\pm$ 11	69 $\pm$ 8
Control 2 hr.	99 $\pm$ 4	49 $\pm$ 4	98 $\pm$ 11	59 $\pm$ 6
Ouabain 2 hr.	98 $\pm$ 5	48 $\pm$ 6	95 $\pm$ 12	58 $\pm$ 5
Control 3 hr.	96 $\pm$ 6	50 $\pm$ 3	100 $\pm$ 9	58 $\pm$ 7
Ouabain 3 hr.	99 $\pm$ 5	51 $\pm$ 5	98 $\pm$ 10	60 $\pm$ 2

<sup>a</sup> Tissues were incubated either in N.K.R. (control) or in the presence of ouabain for indicated length of time at 37°C.

<sup>b</sup> Ouabain concentration was  $5 \times 10^{-8}$  M.

<sup>c</sup> Ouabain concentration was  $10^{-6}$  M.

<sup>d</sup> Na<sup>+</sup> or K<sup>+</sup> content expressed as meq/kg wet weight  $\pm$  S.E.





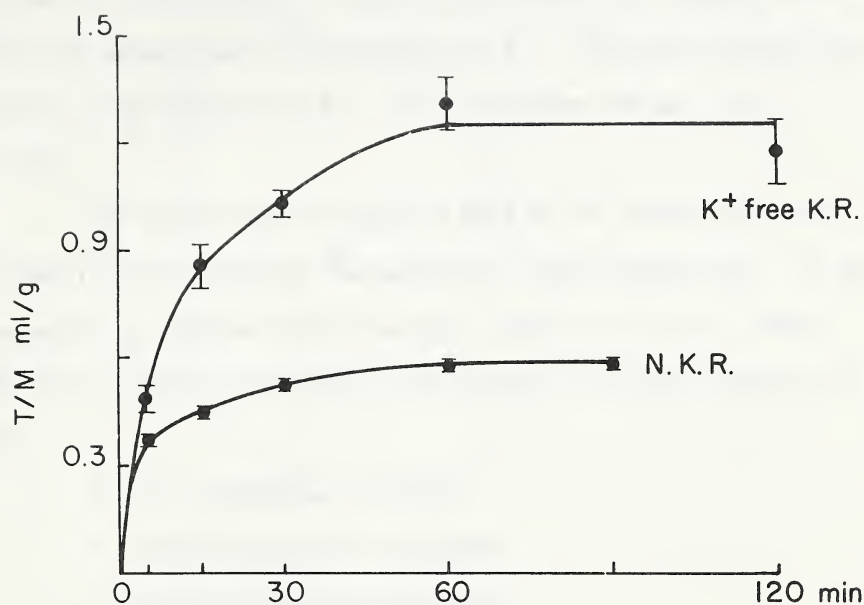


Figure 1. Uptake of  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain by rabbit myometrium in N.K.R. or in  $\text{K}^+$ -free Krebs Ringer at  $37^\circ\text{C}$ . Each point is a mean of six determinations. Vertical bars are S.E.



cardiac glycosides to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  to be inversely related to the external  $\text{K}^+$  concentration. Figure 1 also shows the uptake of  $^3\text{H}$ -ouabain by myometrium in the absence of  $\text{K}^+$ .  $^3\text{H}$ -ouabain uptake was enhanced in the absence of  $\text{K}^+$ . At equilibrium T/M was  $1.3 \pm 0.4$  ml/g.

The above result suggested that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  might be involved in the binding of  $^3\text{H}$ -ouabain to rabbit myometrium. If so, I expected to observe the following: (Baker and Willis, 1969; Dutta et al, 1968 b; Kupfenberg and Schanker, 1968 and Schwartz et al, 1969).

1.  $\text{K}^+$  antagonism to uptake
2. Uptake to be  $\text{Na}^+$ -dependent
3. Uptake to be ATP-dependent
4. Uptake to be temperature sensitive
5. Competition for uptake by other active cardiac

glycosides. (Godfraind and Lesne, 1970 and Kuschinsky et al, 1967, 1968 a).

In addition, if uptake occurred by a single mechanism or binding occurred to a single protein involved in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, the fraction of  $^3\text{H}$ -ouabain affected by other variables in the above list should be the one antagonized by high  $\text{K}^+$  concentrations.

$\text{K}^+$  antagonism: Figure 2 shows the effect of varying concentrations of  $\text{K}^+$  on  $^3\text{H}$ -ouabain uptake by rabbit myometrium. 2.3 mM  $\text{K}^+$  reduced the uptake to almost 50% of the total uptake. 4.5 mM  $\text{K}^+$



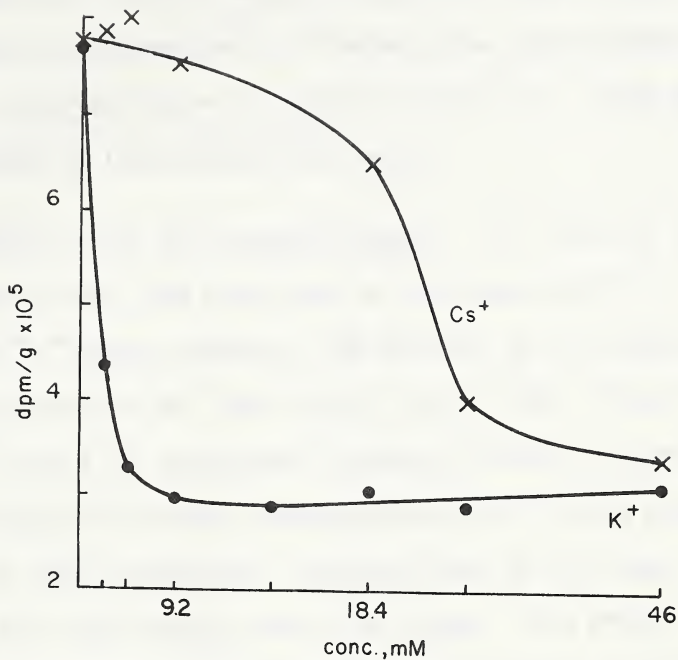


Figure 2. Effect of varying concentrations of  $\text{K}^+$  and  $\text{Cs}^+$  on the uptake of  $5 \times 10^{-8} \text{ M } ^3\text{H-ouabain}$  by rabbit myometrium at  $37^\circ\text{C}$ . Each point is a mean of at least four determinations. The uptake is expressed as dpm/g (wet weight) of tissue. Tissues were incubated in media containing varying concentrations of  $\text{K}^+$  or  $\text{Cs}^+$  for 60 minutes; 60 minutes in media plus  $^3\text{H-ouabain}$ .



(concentration of  $K^+$  in NKR) reduced the uptake by 80% of the total uptake. Higher concentrations of  $K^+$  reduced the uptake which is expressed as dpm/g of tissue, to that for inulin (i.e., that expected for distribution in the extracellular space).

Effect of  $Cs^+$  on  $^3H$ -ouabain uptake:  $Cs^+$ , but only at higher concentrations, has been shown to substitute for  $K^+$  in activating  $Na^+-K^+$ -ATPase (Hoffman, 1965 and Post et al, 1965) and also in reactivation of  $Na^+$  pump (Taylor et al, 1970). Thus I expected  $Cs$ , like  $K^+$  to antagonize  $^3H$ -ouabain binding. Figure 2 shows the effect of different concentrations of  $Cs^+$  on the uptake of  $^3H$ -ouabain by rabbit myometrium. Concentrations of  $Cs^+$  lower than 18.4 mM did not significantly reduce the uptake. 18.4 mM  $Cs^+$  reduced the uptake significantly and 23 mM  $Cs^+$  reduced the uptake maximally, to a T/M ratio like that for inulin.

Uptake of  $^3H$ -ouabain in  $Na^+$ -free medium: Table 2 shows the uptake of  $^3H$ -ouabain in the presence of  $Li^+$  or choline chloride which were used to replace  $Na^+$  in the media. Tissues were preincubated in these media for one hour before the uptake. This period of incubation was found to be sufficient to leach out most of the  $Na^+$  from the tissue (residual  $Na^+$  was 7 to 12 meq/kg wet weight of tissue). As  $Li^+$  has been shown to substitute for  $K^+$  in the activation of  $Na^+-K^+$ -ATPase (Skou, 1960 and Post et al, 1965) choline medium was also used for the uptake. There was only a 25% and statistically





TABLE 2

Uptake of  $^3\text{H}$ -ouabain in  $\text{Na}^+$ -free media<sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	T/M <sup>b</sup> ml/g	P value
1	N.K.R.	$0.58 \pm 0.08$	
2	Choline Ringer	$0.43 \pm 0.08$	$> .1^c$
3	$\text{Li}^+$ Ringer	$0.44 \pm 0.07$	$> .1^c$
4	$\text{K}^+$ -free Ringer	$1.19 \pm 0.11$	
5	$\text{K}^+$ -free Choline Ringer	$0.52 \pm 0.05$	$< .005^d$
6	$\text{K}^+$ -free $\text{Li}^+$ Ringer	$0.50 \pm 0.06$	$< .005^d$

<sup>a</sup> Tissues were incubated for 60 minutes at  $37^\circ\text{C}$  in  $\text{Na}^+$ -free Krebs Ringer, 60 minutes in medium plus  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain.

<sup>b</sup> Mean of 8 determinations  $\pm$  S.E.

<sup>c</sup> P value for difference from control in NKR (No. 1).

<sup>d</sup> P value for difference from control in  $\text{K}^+$ -free Ringer (No. 4).



insignificant decrease ( $P > 0.1$ ) when the uptake was carried out in a  $\text{Na}^+$ -free medium containing 4.6 mM  $\text{K}^+$ . However, when the uptake was carried out in  $\text{K}^+$ -free,  $\text{Li}^+$  or choline Ringer there was significant reduction to a value similar to the inulin T/M ratio (as compared to the uptake in  $\text{K}^+$ -free Ringer (see 'Discussion')). Thus the fraction affected by removal of  $\text{Na}^+$  was the one inhibited by  $\text{K}^+$ .

#### Effect of metabolic inhibitors on the uptake of $^3\text{H}$ -ouabain:

0.2 mM of iodoacetic acid (IAA) or 1 mM of dinitrophenol (DNP) or a mixture of both was used in these experiments. Tissues were pre-incubated for one hour in the presence of these inhibitors. This period of incubation has been shown to be sufficient to deplete ATP stores in the myometrium (Rangachari and Paton, 1970). Table 3 shows the effect of these inhibitors on the uptake of  $^3\text{H}$ -ouabain in NKR. Again, in NKR, the reduction was small and statistically insignificant (5% in the case of DNP and 10% with IAA). However, the effect in  $\text{K}^+$ -free Ringer was about 45% to 58% reduction in the uptake (Table 4) (see 'Discussion')). This reduction was not due to swelling of tissues because the wet weights used were those taken before the addition of inhibitors. The weight gain was 5% to 10% of the original weight. As with removal of  $\text{Na}^+$ , the fraction of  $^3\text{H}$ -ouabain binding sensitive to metabolic inhibitors was the  $\text{K}^+$  sensitive one.

#### Effect of temperature on the uptake and release of previously bound $^3\text{H}$ -ouabain:

Tables 3 and 4 show the uptake of  $^3\text{H}$ -ouabain in NKR and  $\text{K}^+$ -free Ringer at  $4^\circ\text{C}$ . The uptake was about the same as that



TABLE 3

Effect of metabolic inhibitors and temperature on  $^3\text{H}$ -ouabain uptake  
in NKR<sup>a</sup>

No.	Inhibitor	Temp. °C	T/M <sup>b</sup> ml/g	P value <sup>c</sup>
1	-	37	0.76 $\pm$ 0.10	
2	-	4	0.60 $\pm$ 0.01*	< .01
3	DNP $10^{-3}$ M	37	0.72 $\pm$ 0.02*	> .1
4	IAA $2 \times 10^{-4}$ M	37	0.69 $\pm$ 0.06*	> .1

<sup>a</sup> Tissues were incubated for 60 minutes at 37°C in N.K.R. containing metabolic inhibitor, 60 minutes in same media plus  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain.

<sup>b</sup> Mean of 6 determinations  $\pm$  S.E.

<sup>c</sup> P value for difference from control in NKR (No. 1).

\* Differences not significant by Sheffe's test ( $p < 0.05$ ) taking No. 1 as control.



TABLE 4

Effect of metabolic inhibitors and temperature on  $^3\text{H}$ -ouabain uptake in  $\text{K}^+$ -free Ringer

Results are expressed as tissue to medium ratio (ml/g wet weight).

No.	Inhibitor	Temp. $^{\circ}\text{C}$	T/M <sup>b</sup> ml/g	P value <sup>c</sup>
1	-	37	$1.07 \pm 0.16$	
2	-	4	$0.42 \pm 0.06^*$	< .01
3	DNP $10^{-3}$ M	37	$0.66 \pm 0.07^*$	< .05
4	IAA $2 \times 10^{-4}$ M	37	$0.50 \pm 0.04^*$	< .01
5	IAA + DNP	37	$0.37 \pm 0.01^*$	< .005

<sup>a</sup> Tissues were incubated for 60 minutes at  $37^{\circ}\text{C}$  in  $\text{K}^+$ -free Ringer containing metabolic inhibitor, 60 minutes in same media plus  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain.

<sup>b</sup> Mean of 6 determinations  $\pm$  S.E.

<sup>c</sup> P values for difference from control in  $\text{K}^+$ -free Ringer (no. 1).

\* Differences found significant using Sheffe's test ( $p < 0.05$ ) taking No. 1 as control.





for inulin. Omission of  $K^+$  at this temperature did not alter the uptake, suggesting a high temperature dependence for  $K^+$  sensitive uptake.

The temperature dependence of release of  $^3H$ -ouabain was also studied (Table 5). There was significantly more residual  $^3H$ -ouabain still bound after 90 minutes at  $37^{\circ}C$  when efflux was into  $K^+$ -free solution; however, if  $10^{-5}$  M unlabelled ouabain was present in the efflux media (to increase efflux by exchange if this occurs), omission of  $K^+$  did not affect residual  $^3H$ -ouabain. At  $4^{\circ}C$  the presence or absence of  $K^+$  or ouabain had no significant effect and obviously much less  $^3H$ -ouabain was released. Thus efflux of  $^3H$ -ouabain was highly temperature dependent and this temperature dependent release was accelerated by  $K^+$  and ouabain.

When the uptake occurred in the presence of 18.4 mM  $K^+$  and tissues were washed subsequently for 90 minutes at  $37^{\circ}C$  or  $4^{\circ}C$  (Table 6) there was in both cases only a small amount of residual  $^3H$ -ouabain (compare Tables 5 and 6) and the differences in residual  $^3H$ -ouabain bound at the end of 90 minutes were smaller but significant. Addition of ouabain had no effect on the residual  $^3H$ -ouabain. This suggests that a fraction of the  $K^+$ -independent ouabain binding was also temperature dependent.

Effect of digitoxin on  $^3H$ -ouabain uptake: Table 7 shows the effect of varying concentrations of digitoxin on the uptake. Inhibition of  $^3H$ -ouabain uptake was not so clearly seen in NKR and



TABLE 5

Release of previously bound  $^3\text{H}$ -ouabain<sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	Temp. °C <sup>b</sup>	T/M <sup>c</sup> ml/g	P value
1	K <sup>+</sup> -free Ringer (uptake)	-	1.60 ± 0.24	
2	NKR	37	0.174 ± 0.011	< .025 <sup>e</sup>
3	NKR + 10 <sup>-5</sup> M ouabain	37	0.136 ± 0.013	
4	K <sup>+</sup> -free	37	0.255 ± 0.330	< .01 <sup>f</sup>
5	K <sup>+</sup> -free + 10 <sup>-5</sup> M ouabain	37	0.144 ± 0.013	
6	NKR	4	0.98 ± 0.11	> .05 <sup>g</sup>
7	NKR + 10 <sup>-5</sup> M ouabain	4	1.20 ± 0.29	> .1 <sup>g</sup>
8	K <sup>+</sup> -free	4	1.0 ± 0.17	> .1 <sup>g</sup>
9	K <sup>+</sup> -free + 10 <sup>-5</sup> M ouabain	4	1.08 ± 0.35	> .1 <sup>g</sup>

<sup>a</sup> Tissues were incubated for 60 minutes in K<sup>+</sup>-free Krebs Ringer at 37°C in the presence of  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M) and then washed for 90 minutes in indicated medium at 37°C or 4°C. Washing medium was changed every 10 minutes for the first 30 minutes and every 30 minutes thereafter.

<sup>b</sup> Temperature of washing medium.

<sup>c</sup> Mean of 8 tissues ± S.E.

<sup>d</sup> P value for difference between No. 2 and No. 4.

<sup>e</sup> P value for difference between No. 2 and No. 3.

<sup>f</sup> P value for difference between No. 4 and No. 5.

<sup>g</sup> P value for difference from control (No. 1).



TABLE 6

Release of  $^3\text{H}$ -ouabain taken up in Krebs Ringer containing 18.4 mM  $\text{K}^+$ <sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	Temp. $^{\circ}\text{C}$	T/M ml/g <sup>c</sup>	P value
1	- <sup>b</sup>		0.53 $\pm$ 0.07	
2	NKR	37	0.040 $\pm$ 0.009	< .005 <sup>d</sup>
3	NKR	4	0.093 $\pm$ 0.002	
4	NKR + $10^{-5}$ M ouabain	37	0.034 $\pm$ 0.009	< .01 <sup>e</sup>
5	NKR + $10^{-5}$ M ouabain	4	0.081 $\pm$ 0.008	

<sup>a</sup> Tissues were incubated for 60 minutes in 18.4 mM  $\text{K}^+$  Ringer at  $37^{\circ}\text{C}$  in the presence of  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M) and then washed in indicated media for 90 minutes at  $37^{\circ}\text{C}$  or  $4^{\circ}\text{C}$ .

<sup>b</sup> Uptake in 18.4 mM  $\text{K}^+$  Ringer.

<sup>c</sup> Mean of 8 determinations  $\pm$  S.E.

<sup>d</sup> P value for difference between No. 2 and No. 3.

<sup>e</sup> P value for difference between No. 4 and No. 5.



TABLE 7

Effect of digitoxin on  $^3\text{H}$ -ouabain uptake<sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Concentration of digitoxin (M)	T/M <sup>b</sup> ml/g			
		Control	NKR	+ digitoxin	Control K <sup>+</sup> -free Ringer
1	$5 \times 10^{-8}$	$0.85 \pm 0.14$		$0.83 \pm 0.13$	$1.99 \pm 0.11$
2	$10^{-7}$	$0.75 \pm 0.09$		$0.75 \pm 0.10$	$1.60 \pm 0.17$
3	$10^{-6}$	$0.75 \pm 0.08$		$0.73 \pm 0.08$	$1.20 \pm 0.04$
4	$10^{-5}$	$0.85 \pm 0.14$		$0.70 \pm 0.12$	$1.71 \pm 0.06$
					$1.75 \pm 0.10$
					$0.90 \pm 0.07$
					$0.70 \pm 0.04$
					$0.55 \pm 0.04$

<sup>a</sup> Tissues were incubated for 60 minutes in NKR or K<sup>+</sup>-free Ringer at 37°C in the presence of different concentrations of digitoxin, 60 minutes in similar media plus  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain.

<sup>b</sup> Mean of 6 determinations  $\pm$  S.E.





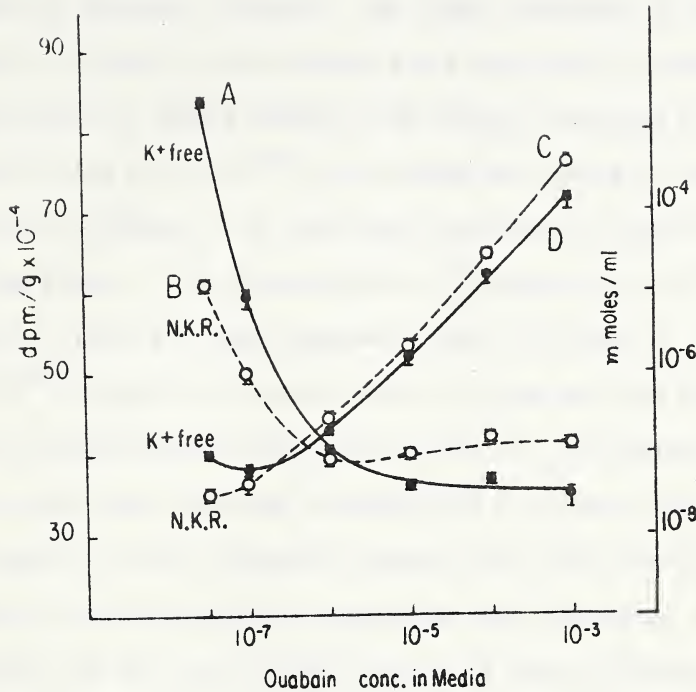
was only about 27% even when  $10^{-5}$  M digitoxin was used (see 'Discussion'). However, the inhibition was obvious in  $K^+$ -free Ringer. Equimolar concentrations of digitoxin ( $5 \times 10^{-8}$  M) did not reduce the uptake, but higher concentrations significantly reduced the uptake.  $10^{-5}$  M digitoxin reduced  $^3H$ -ouabain uptake to a value similar to that of inulin. Thus the fraction of  $^3H$ -ouabain binding reduced by digitoxin was the  $K^+$ -sensitive one.

Effect of unlabelled ouabain on the uptake of  $^3H$ -ouabain:

Figure 3 compares the effect of varying concentrations of unlabelled ouabain on the uptake of  $^3H$ -ouabain in NKR and  $K^+$ -free Ringer. Counts per minute in the incubation media were kept constant by using a constant volume of medium containing  $^3H$ -ouabain and reducing the specific activity by adding required amount of unlabelled ouabain. The concentration of  $^3H$ -ouabain was  $5 \times 10^{-8}$  M.  $10^{-6}$  M ouabain reduced the uptake of labelled ouabain to that of inulin.

In  $K^+$ -free Ringer the uptake of radioactive ouabain is higher at  $5 \times 10^{-8}$  M and  $10^{-7}$  M. In Figure 3 the cellular uptake expressed as moles/ml of cell water (calculated by subtracting ouabain concentration in the inulin space from the total uptake) at equilibrium was plotted against the ouabain concentration in incubation media. The uptake, thus calculated, was almost the same at  $5 \times 10^{-8}$  M and  $10^{-7}$  M of ouabain concentration. In  $K^+$ -free Ringer the amount taken up at  $5 \times 10^{-8}$  M is higher than the amount taken up in NKR. At ouabain concentrations above  $10^{-7}$  M the amount





**Figure 3.** Comparison of the effect of varying concentrations of unlabelled ouabain on the uptake of  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain in NKR and  $\text{K}^+$ -free Ringer at  $37^\circ\text{C}$ . The uptake of  $^3\text{H}$ -ouabain is expressed as dpm/g (wet weight) of tissue, A & B. The total uptake of ouabain is expressed as mmoles/ml of cell water, C & D. Each point is a mean of six determinations. Vertical bars are S.E. and when absent are within the point. Ouabain concentrations in the media are on a molar basis. Tissues were incubated in the presence of varying concentrations of unlabelled ouabain in  $\text{K}^+$ -free Ringer or NKR for 60 minutes; 60 minutes in media plus  $^3\text{H}$ -ouabain.



taken up increases linearly. The linear component of total ouabain uptake is probably a non-saturable and non-specific uptake of ouabain. As the specific uptake seemed to be getting saturated at an ouabain concentration of  $5 \times 10^{-8}$  M, we studied the uptake of lower concentrations of ouabain in an additional experiment (Figure 4) in  $K^+$ -free Ringer. The concentration of  $^3H$ -ouabain was  $10^{-9}$  M hence the dpm/g of tissue are less compared to that of Figure 3. Up to  $5 \times 10^{-8}$  M ouabain the uptake curve is linear and the ATPase sites seem to reach complete saturation at  $10^{-7}$  M. The radioactivity curve shows that below the concentration of ouabain that saturates the specific sites, unlabelled ouabain has little effect on the uptake of labelled ouabain, suggesting that the number of available binding sites was not limiting binding at these concentrations. The total uptake in Figure 4 is lower than that found in Figure 3. This is probably due to experimental variation in uptake in different experiments.

#### UPTAKE OF $^3H$ -OUABAIN BY RAT MYOMETRIUM

The rate of uptake in NKR: As in the rabbit myometrium an equilibrium was reached within 60 minutes for uptake of ouabain from a concentration of  $10^{-6}$  M. The T/M was  $0.49 \pm 0.03$  ml/g at equilibrium which is slightly lower than that found with rabbit tissue. This is shown in Table 8.



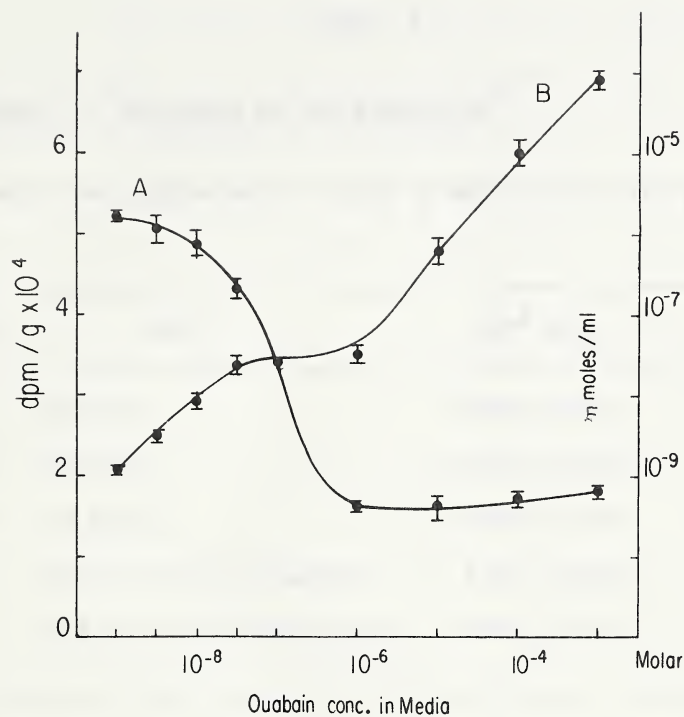


Figure 4. Effect of varying concentrations of unlabelled ouabain on the uptake of  $10^{-9}$  M  $^3\text{H}$ -ouabain in  $\text{K}^+$ -free Ringer at  $37^\circ\text{C}$ . Conditions are as in Figure 3, each point is a mean of six determinations. Vertical bars are S.E.





TABLE 8

Uptake of  $^3\text{H}$ -ouabain by rat myometrium<sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	$\bar{T}/M^b$ ml/g	P value <sup>c</sup>
1	N.K.R.	$0.49 \pm 0.03$	
2	$\text{K}^+$ -free	$0.53 \pm 0.02$	> .1
3	46 mM $\text{K}^+$	$0.50 \pm 0.04$	> .1
4	N.K.R. + $10^{-3}$ M ouabain	$0.51 \pm 0.03$	> .1
5	N.K.R. + $10^{-4}$ M digitoxin	$0.48 \pm 0.05$	> .1

<sup>a</sup> Tissues were incubated in indicated media at  $37^\circ\text{C}$  for 60 minutes, 60 minutes in media plus  $10^{-6}$  M  $^3\text{H}$ -ouabain.

<sup>b</sup> Mean of 15 determinations  $\pm$  S.E.

<sup>c</sup> P value for difference from control in N.K.R.



The uptake of ouabain in  $K^+$ -free and high  $K^+$  media: Omission of  $K^+$  did not alter the uptake of  $^3H$ -ouabain (Table 8). Effect of 46 mM  $K^+$  on the uptake is also shown in Table 8. High  $K^+$  did not reduce the T/M for ouabain. The concentration used in these experiments was  $10^{-6}$  M ouabain in the case of rats which is 500 times the concentration used in rabbits. Possibly the lack of any effect of  $K^+$  resulted from saturation of ouabain binding sites at that concentration; (see Akera et al, 1971; Hoffman, 1969, and Landowne and Ritchie, 1970) so that the observed uptake was at non-specific sites. In order to eliminate this possibility we repeated the experiment with  $5 \times 10^{-8}$  M ouabain, the concentration used in the case of rabbits. Again there was no difference in the uptake either in  $K^+$ -free Ringer or high  $K^+$  Ringer (Table 9). On the other hand, the affinity of ouabain for binding sites in this tissue may be so low that  $10^{-6}$  M does not bind to  $Na^+-K^+$ -ATPase sites. In order to eliminate this doubt uptakes were carried out in concentrations of  $^3H$ -ouabain ranging from  $10^{-6}$  M to  $10^{-3}$  M in NKR and  $K^+$ -free Krebs Ringer. Again no significant difference in the T/M ratio was seen between the two groups (Table 10).

The effect of digitoxin and unlabelled ouabain on the uptake: Table 8 also shows that neither  $10^{-4}$  M digitoxin nor  $10^{-3}$  M ouabain had any effect on the uptake of  $^3H$ -ouabain by rat myometrium.



TABLE 9

Uptake of  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain by rat myometrium

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	T/M <sup>a</sup> ml/g	P value <sup>b</sup>
1	N.K.R.	$0.61 \pm 0.09$	
2	K <sup>+</sup> -free Ringer	$0.62 \pm 0.08$	> .1

<sup>a</sup> Mean of 8 determinations  $\pm$  S.E.

<sup>b</sup> P value for difference from control in N.K.R.



TABLE 10

Uptake of various concentrations of  $^3\text{H}$ -ouabain by rat myometrium<sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g) wet weight

Conc. (M)	N.K.R. <sup>b</sup>	K <sup>+</sup> -free K.R. <sup>b</sup>	P value <sup>c</sup>
10 <sup>-3</sup>	0.54 $\pm$ 0.017	0.49 $\pm$ 0.047	> .1
10 <sup>-4</sup>	0.61 $\pm$ 0.057	0.46 $\pm$ 0.031	> .1
10 <sup>-5</sup>	0.69 $\pm$ 0.54	0.49 $\pm$ 0.026	> .1
10 <sup>-6</sup>	0.57 $\pm$ 0.017	0.63 $\pm$ 0.047	> .1

<sup>a</sup> Tissues were incubated in media containing indicated concentration of ouabain at 37°C for 60 minutes.

<sup>b</sup> Mean of 6 determinations.

<sup>c</sup> P value for difference between N.K.R. and K<sup>+</sup>-free K.R.





The uptake by rat myometrium was also not affected by either metabolic inhibitors or  $\text{Na}^+$ -free media (Data not shown).

#### THE UPTAKE OF $^3\text{H}$ -DIGITOXIN

The rate of uptake in NKR: The rate of uptake was similar for both rabbit and rat myometrium. This is shown in Figure 5. Equilibrium was reached within 60 minutes. The T/M was much higher than that for  $^3\text{H}$ -ouabain in both cases. It was  $2.8 \pm 0.12$  ml/g for rabbit and  $3.0 \pm 0.14$  ml/g for rat tissue.

The rate of uptake in  $\text{K}^+$ -free Ringer: Figure 5 also shows that omission of  $\text{K}^+$  did not affect either the rate or the final T/M at the end of 60 minutes in rabbit myometrium. We thought that the failure to see the effect of  $\text{K}^+$  was due to masking of the specific uptake by the high non-specific uptake of  $^3\text{H}$ -digitoxin related to its lipid solubility. To minimize non-specific uptake tissues were incubated for shorter lengths of time, i.e., for 5, 15 and 30 minutes. Also, as there was variation in the T/M from one tissue piece to another we used paired controls, i.e., each myometrial piece was cut into two halves longitudinally. These pieces were used to compare the uptake in NKR and  $\text{K}^+$ -free Ringer. As it is shown in Table 11, no significant difference was seen between the uptake in NKR and  $\text{K}^+$ -free Ringer.



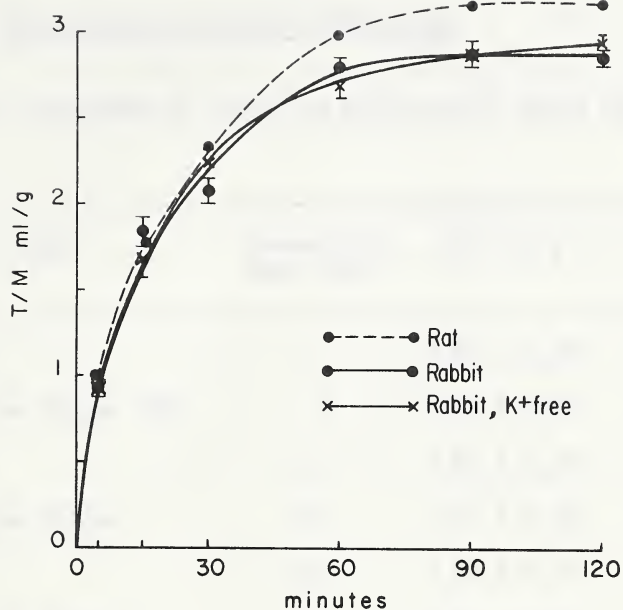


Figure 5. Uptake of  $5 \times 10^{-8}$  M  $^3\text{H}$ -digitoxin by rabbit and rat myometrium at  $37^{\circ}\text{C}$ . Tissues were incubated in N.K.R. or  $\text{K}^{+}$ -free Ringer. Each point is a mean of six determinations. Vertical bars are S.E.



TABLE 11

Uptake of  $^3\text{H}$ -digitoxin by rabbit myometrium<sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	Incubation time (min)	T/M <sup>b</sup> ml/g	P value <sup>c</sup>
1	NKR	5	0.81 $\pm$ 0.04	> .1
2	K <sup>+</sup> -free Ringer NKR	5	0.69 $\pm$ 0.05	
3	NKR	15	1.81 $\pm$ 0.32	> .1
4	K <sup>+</sup> -free Ringer	60	1.97 $\pm$ 0.06	
5	NKR	60	3.20 $\pm$ 0.14	> .1
6	K <sup>+</sup> -free Ringer	60	3.01 $\pm$ 0.60	

<sup>a</sup> Tissues were incubated in NKR or K<sup>+</sup>-free Krebs Ringer at 37°C for 60 minutes, 60 minutes in medium plus  $5 \times 10^{-8}$  M  $^3\text{H}$ -digitoxin.

<sup>b</sup> Mean of 12 determinations  $\pm$  S.E.

<sup>c</sup> P value for difference from control in N.K.R. (Paired controls)



The effect of high  $K^+$ : 46 mM  $K^+$  had no effect on  $^3H$ -digitoxin uptake by rabbit myometrium. The uptake was also not reduced in  $Na^+$ -free media, by metabolic inhibitors, unlabelled digitoxin or  $10^{-5}$  M ouabain (Table 12 and Table 13).

The release of previously bound  $^3H$ -digitoxin: When tissues were incubated in  $K^+$ -free Ringer or 18.4 mM  $K^+$  media and then washed for 90 minutes in  $K^+$ -free Ringer or NKR, there was no difference in the amount bound between the tissue incubated in  $K^+$ -free Ringer and 18.4 mM  $K^+$  media (Table 14). Also washing in either  $K^+$ -free Ringer or NKR did not change the amount of residual  $^3H$ -digitoxin.

## DISCUSSION

The results showed that  $5 \times 10^{-8}$  M ouabain in rabbit or  $10^{-6}$  M in rat uterine tissue did not cause any loss of  $K^+$  or gain of  $Na^+$  over the three-hour incubation period. Therefore, there was no appreciable inhibition of the  $Na^+$  pump at these concentrations. Daniel (1964 a,b) also found that  $10^{-6}$  M ouabain did not cause any gain in  $Na^+$  in non-pregnant rat myometrium. The reason for using these two concentrations was to have minimal inhibition of the  $Na^+$  pump which was justified by the results.

One fraction of the uptake of  $^3H$ -ouabain by rabbit myometrium behaves experimentally exactly as predicted if binding involved





TABLE 12

Effect of 46 mM K<sup>+</sup>, omission of Na<sup>+</sup> and effect of metabolic inhibitors on the uptake of  $5 \times 10^{-8}$  M <sup>3</sup>H-digitoxin

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Media	T/M <sup>a</sup> ml/g	P value <sup>b</sup>
1	N.K.R.	2.8 $\pm$ 0.12	
2	IAA $2 \times 10^{-4}$ M	3.0 $\pm$ 0.09	> .1
3	DNP $10^{-3}$ M	2.8 $\pm$ 0.11	> .1
4	Choline Ringer	2.7 $\pm$ 0.14	> .1
5	46 mM K <sup>+</sup>	3.0 $\pm$ 0.10	> .1

<sup>a</sup> Mean of 6 determinations  $\pm$  S.E.

<sup>b</sup> P value for difference from control in N.K.R.



TABLE 13

Effect of unlabelled digitoxin ( $10^{-5}$  M) and ouabain ( $10^{-5}$  M) on the uptake of  $5 \times 10^{-8}$  M  $^3\text{H}$ -digitoxin

No.	Media	T/M <sup>a</sup> ml/g	P value <sup>b</sup>
1	N.K.R.	$3.0 \pm 0.20$	
2	K <sup>+</sup> -free Ringer	$3.1 \pm 0.18$	> .1
3	N.K.R. + $10^{-5}$ M ouabain	$2.9 \pm 0.18$	> .1
4	K <sup>+</sup> -free Ringer + $10^{-5}$ M ouabain	$3.3 \pm 0.14$	> .1
5	N.K.R. + $10^{-5}$ M digitoxin	$3.2 \pm 0.12$	> .1

<sup>a</sup> Mean of 6 determinations  $\pm$  S.E.

<sup>b</sup> P value for difference from control in N.K.R.



TABLE 14

Release of previously bound  $^3\text{H}$ -digitoxin at  $37^\circ\text{C}$ <sup>a</sup>

Results are expressed as tissue to medium ratio (ml/g wet weight)

No.	Uptake Media	Washing Media	T/M <sup>b</sup> ml/g	P value <sup>c</sup>
1	18.4 mM $\text{K}^+$ Ringer	-	5.2 0.34	
2	18.4 mM $\text{K}^+$ Ringer	$\text{K}^+$ -free Ringer	0.45 0.11	> .1
3	18.4 mM $\text{K}^+$ Ringer	N.K.R.	0.58 0.13	
4	$\text{K}^+$ -free Ringer	-	6.0 0.32	
5	$\text{K}^+$ -free Ringer	$\text{K}^+$ -free Ringer	0.60 0.12	> .1
6	$\text{K}^+$ -free Ringer	N.K.R.	0.58 0.09	

<sup>a</sup> Tissues were incubated in 18.4 mM  $\text{K}^+$  Ringer or  $\text{K}^+$ -free Ringer at  $37^\circ\text{C}$  for 60 minutes, 60 minutes in media plus  $5 \times 10^{-8}$  M  $^3\text{H}$ -digitoxin and then washed for 90 minutes in indicated media.

<sup>b</sup> Mean of 5 determinations  $\pm$  S.E.

<sup>c</sup> P value for difference between No. 2 and No. 3 and No. 5 and No. 6.



$\text{Na}^+$ - $\text{K}^+$ -activated ATPase, i.e., it was dependent on  $\text{Na}^+$  and ATP and antagonized by  $\text{K}^+$ . The behaviour of this fraction is also consistent with the reports of ouabain binding to red cell ghost membranes (Dunham and Hoffman, 1970), HeLa cells, (Baker and Willis, 1969) non-myelinated nerve fibres, (Landowne and Ritchie, 1970) microsomal fraction of heart, (Dutta et al, 1968 a,b, 1969; Prindle et al, 1971 and Matsui and Schwartz, 1968) and binding to  $\text{Na}^+$ - $\text{K}^+$ -ATPase preparation of kidney (Akera and Brody, 1971; Hansen, 1971 and Schwartz et al, 1971, and Tobin and Sen, 1970), electrophorus electric organ (Albers et al, 1968). These workers showed  $\text{K}^+$  antagonism to ouabain binding, which may be related to the antagonism of ouabain inhibition of  $\text{Na}^+$ - $\text{K}^+$ -ATPase. Elevated  $\text{K}^+$  overcomes ouabain inhibition of  $\text{Na}^+$  pump in red cells (Glynn, 1957, Hoffman, 1962) and also inhibition of  $\text{Na}^+$ - $\text{K}^+$ -ATPase, (Akera and Brody, 1969; Judah and Ahmed, 1966; Matsui and Schwartz, 1966, Post et al, 1965; Schatzmann, 1963 and Yoda and Hokin, 1970). Landowne and Ritchie (1970) found that in the presence of high external  $\text{K}^+$ , ouabain inhibition of the pump in non-myelinated nerve fibres was significantly lowered. Matsui and Schwartz (1968) have shown that binding of cardiac glycosides to ATPase and inhibition of ATPase are related (see also Barnett, 1970).

The uptake of ouabain by rabbit myometrium was  $\text{Na}^+$  dependent. The effect of  $\text{Na}^+$  removal in Krebs Ringer containing 4.6 mM  $\text{K}^+$  was smaller than in  $\text{K}^+$ -free and  $\text{Na}^+$ -free Ringer. The  $\text{K}^+$





present in NKR (4.6 mM) reduces uptake by about 80% (see Figure 2) and thus effect of  $\text{Na}^+$  removal from NKR on the uptake is insignificant. This supports that the  $\text{K}^+$  sensitive binding is the one which is  $\text{Na}^+$  dependent. The same argument holds true for the effect of metabolic inhibitors, of digitoxin and of unlabelled ouabain on the uptake of  $^3\text{H}$ -ouabain in NKR. This is also probably the reason for the results of Greenberger et al, (1969) who reported no specific uptake for ouabain by everted intestinal loops. The concentration of  $\text{K}^+$  was 6.2 mM in their media.

$\text{Na}^+$  dependence for ouabain binding to HeLa cells has been shown by Baker and Willis (1969) and to microsomal fractions of guinea pig heart and  $\text{Na}^+-\text{K}^+$ -ATPase of dog heart by Dutta and Marks (1969) and Schwartz et al, (1968) respectively.  $\text{Na}^+$  is required for the activation of  $\text{Na}^+-\text{K}^+$ -ATPase intracellularly as shown in the studies with erythrocyte ghost (Glynn, 1962 and Whittam, 1962). These results are consistent with these findings. Tobin and Sen (1970) have shown that in the presence of  $\text{Mg}^{++}$  and ATP,  $\text{Na}^+$  stimulates ouabain binding presumably to the  $\text{E}_2 - \text{P}$  form of the enzyme which is the conformationally altered phospho-enzyme. The uptake conditions of our experiments probably facilitate this type of binding.

Baker and Willis (1969) and Kupfenberg and Schanker (1968) who studied ouabain binding to HeLa cells and liver slices respectively, found that this binding was ATP-dependent. They found that metabolic inhibitors like DNP or  $\text{CN}^-$  prevented ouabain binding. Accumulation



of ouabain and digitoxin by guinea pig hearts was shown to be energy dependent by Dutta et al (1972). Binding of ouabain to red cell ghosts was also ATP-dependent (Hoffman, 1969). Studies with isolated enzyme also have shown that ouabain binding to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is ATP-dependent (Albers et al, 1968; Matsui and Schwartz, 1967, 1968; Schwartz et al, 1968 and Tobin and Sen, 1970). Present results also show that  $\text{K}^+$  sensitive ouabain binding to rabbit myometrium was ATP-dependent. Daniel and Robinson (1971 b) and Rangachari and Paton (1970) have shown that incubation of myometrium with IAA or IAA + DNP for 60 minutes depletes the ATP stores almost completely. DNP reduced the ATP stores only by 50% (Daniel and Robinson, 1971 b). This might probably be the reason for the smaller effect of DNP on ouabain binding.

This  $\text{K}^+$  sensitive uptake of ouabain was also temperature sensitive. This agrees with the results of Baker and Willis (1969). Release of  $^3\text{H}$ -ouabain from the myometrium was also temperature dependent. Release of the  $\text{K}^+$  sensitive fraction of uptake was more temperature dependent than the release of  $\text{K}^+$  insensitive fraction of uptake. This agrees with the results of Sen (1969) and Tobin and Sen (1970) who studied the dissociation of ouabain  $\text{Na}^+\text{-K}^+\text{-ATPase}$  complex at different temperatures and found that the complex was very stable at  $0^\circ\text{C}$ . Schwartz et al, (1968) also showed that the complex was stable at  $0^\circ\text{C}$ . However, Albers et al, (1968) and Yoda and Hokin (1970) reported that this binding of ouabain to  $\text{Na}^+\text{-K}^+\text{-ATPase}$



is irreversible. This differs from **present** findings and the reason might be the difference in experimental conditions and the tissue used. The higher residual of  $^3\text{H}$ -ouabain bound in the case of  $\text{K}^+$  insensitive uptake at  $4^\circ\text{C}$  compared to that at  $37^\circ\text{C}$  is probably due to a reduction in the rate of passive diffusion. The release of  $\text{K}^+$  sensitive fraction in intact tissues was accelerated by  $\text{K}^+$  and unlabelled ouabain contrary to the results of Akera and Brody (1971) and Allen et al (1971) who found that  $\text{K}^+$  stabilized the ouabain-enzyme complex. The isolated enzyme might behave differently than in the intact tissue.

The uptake of  $^3\text{H}$ -ouabain was reduced by  $\text{Cs}^+$ , the cation which can substitute for  $\text{K}^+$  in stimulating  $\text{Na}^+-\text{K}^+$ -ATPase (Post et al, 1965 and Skou, 1960). A higher (18.4 mM) concentration than that of  $\text{K}^+$  was required to reduce the ouabain uptake. This was also the concentration required to reactivate partially the  $\text{Na}^+$  pump in rat myometrium (Taylor et al, 1971). These results do not agree with that of Hoffman (1960) who found that  $\text{Cs}^+$  reduced the non-specific binding of ouabain to red cell ghosts.

The uptake of ouabain at the  $\text{Na}^+-\text{K}^+$ -ATPase sites has been shown to be competed for by other active glycosides (Baker and Willis, 1969 and Matsui and Schwartz, 1967). Kupfenberg and Schanker (1968), Kuschinsky et al, (1968) and Godfraind and Lesne (1970) obtained similar results using liver slices, heart and intestinal smooth muscle respectively. My results agree with the above findings.





These  $K^+$  sensitive sites are also saturable. The sites get fully saturated at a concentration of  $10^{-7}$  M. Also the uptake is  $K^+$  sensitive only below this concentration. This was clearly seen when uptake in  $K^+$ -free Ringer with varying concentrations of ouabain was compared with that in NKR (Figure 3). This is consistent with the results of Landowne and Ritchie (1970) who found that above  $20 \mu\text{M}$  of external ouabain concentration the binding to non-myelinated nerve fibres was insensitive to  $K^+$ . They concluded that this  $K^+$  sensitive binding was the one related to inhibition of  $\text{Na}^+$ -pump. This also may be the reason for the results of Forth et al, (1969) who used a concentration of  $10^{-6}$  M and above in guinea pig intestine and reported that there was no uptake of limited capacity. These results justify the use of a concentration below  $10^{-7}$  M in present experiments where the non-specific uptake is at a minimal level.

All these results favour the view that  $\text{Na}^+$ - $K^+$ -ATPase might be the receptor for cardiac glycoside uptake by rabbit myometrium. However, the results with rat myometrium did not agree with our findings in rabbit. The T/M in NKR was lower in rat tissue than that in rabbit tissue and close to the value for inulin. This is consistent with the results of Dutta et al, (1968) and Fujino et al, (1969) in heart. Higher concentrations ( $10^{-6}$  M to  $10^{-3}$  M) of ouabain in the case of rat were used because enzyme studies show that ATPase from the rat tissue is less sensitive (Akeru et al, 1969; Allen and Daniel, 1970; Allen and Schwartz, 1969 and Repke, 1963)





and  $10^{-3}$  M ouabain is required for complete inhibition of the enzyme and the  $\text{Na}^+$  pump (Daniel, 1964 a,b).

Absence of  $\text{K}^+$  antagonism to  $^3\text{H}$ -ouabain binding in the case of rats was unexpected. From enzyme studies it is evident (Akera et al, 1969, Schwartz et al, 1969) that though the enzyme from other rat tissues is insensitive to ouabain and the ouabain enzyme complex less stable, as compared to enzyme from rabbit or guinea pig tissue,  $\text{K}^+$  interaction with the enzyme in overcoming ouabain inhibition is similar. However, elevation of  $\text{K}^+$  did not reverse ouabain inhibition of  $^{22}\text{Na}$  efflux from rat myometrium (Daniel and Robinson, 1971 a). Allen and Daniel (1970) did not find any  $\text{Na}^+$  or  $\text{K}^+$  stimulation of the ATPase from rat myometrium, but Kidwai (unpublished data) recently found a membrane ATPase from rat myometrium which is stimulated by  $\text{K}^+$  and  $\text{Na}^+$  and inhibited by ouabain. From the present results it is difficult to explain these discrepancies. Either the specific binding sites for ouabain on  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  are absent or undetectable in rat tissue or they lack  $\text{K}^+$ -sensitivity in ouabain binding.

The uptake was neither  $\text{Na}^+$  nor ATP-dependent and it was also not reduced either by digitoxin or unlabelled ouabain. One possibility is that the number of ATPase binding sites for ouabain in rat (those which are  $\text{K}^+$  sensitive and  $\text{Na}^+$  and ATP-dependent) must be extremely small and hence undetectable by the present method where uptake into extracellular space cannot be prevented.



This possibility was tested and is described in the next chapter where binding to subcellular fractions is studied.

Uptake of  $^3\text{H}$ -digitoxin by rabbit and rat myometrium was similar. The T/M for digitoxin was much higher than that for ouabain presumably because of higher lipid solubility of digitoxin enables it to cross the plasma membrane. These results are consistent with those of Dutta et al, (1968); Fujino et al, (1969); Godfraind and Lesne (1968), and Kuschinsky et al, (1968) in this respect.

Omission of  $\text{K}^+$  did not alter the uptake of digitoxin, neither did high  $\text{K}^+$  reduce it. Harvey and Peiper in 1955 reported insignificant effects of  $\text{K}^+$  on subcellular distribution of  $^3\text{H}$ -digitoxin. Dutta et al, (1968) also found similar results with isolated sarcoplasmic reticulum of beef and guinea pig hearts. In their report ouabain binding was affected to a greater extent by  $\text{K}^+$  than was digitoxin binding. This is surprising because digitoxin has the same effects on  $\text{Na}^+-\text{K}^+-\text{ATPase}$  as ouabain (Albers et al, 1968 and Repke, 1961) and also  $10^{-5}$  M digitoxin like  $10^{-5}$  M ouabain inhibited the uptake of  $^3\text{H}$ -ouabain in the present studies both in NKR and  $\text{K}^+$ -free Ringer, showing almost the same affinity for  $\text{K}^+$  sensitive ouabain binding sites. However, ouabain or unlabelled digitoxin did not reduce the uptake of  $^3\text{H}$ -digitoxin either in NKR or  $\text{K}^+$ -free Ringer. Godfraind and Lesne (1970) have reported a reduction of  $^3\text{H}$ -digitoxin uptake by ouabain in intestinal smooth muscle, though ouabain did not reduce the uptake of digitoxin to



the same extent as did digitoxin with ouabain uptake. The reasons for this difference between present results and theirs are unclear.

The reason for the failure to see a  $\text{Na}^+\text{-K}^+\text{-ATPase}$  dependent  $^3\text{H}$ -digitoxin uptake is probably due to its very high non-specific non-saturable uptake related to its lipid solubility (see Godfraind and Lesne, 1970). The specific uptake is very small compared to this non-specific uptake, and is probably obscured. But the effect of unlabelled digitoxin on  $^3\text{H}$ -ouabain uptake suggests indirectly at least that it does bind to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  dependent sites. Studies on binding to isolated plasma membrane to prove this, are presented in Chapter IV.



## CHAPTER IV

### SUBCELLULAR DISTRIBUTION OF $^3\text{H}$ -OUABAIN AND $^3\text{H}$ -DIGITOXIN IN RABBIT AND RAT MYOMETRIUM AND BINDING OF THESE GLYCOSIDES TO ISOLATED PLASMA MEMBRANE PREPARATION





## CHAPTER IV

SUBCELLULAR DISTRIBUTION OF  $^3\text{H}$ -OUABAIN AND  $^3\text{H}$ -DIGITOXIN IN RABBIT AND RAT MYOMETRIUM AND BINDING OF THESE GLYCOSIDES TO ISOLATED PLASMA MEMBRANE PREPARATION

## MATERIALS AND METHODS

## Subcellular Fractionation

Myometrial pieces after incubation for 60 minutes in the presence of radioactive glycosides were washed in cold Krebs medium for 10 minutes and then transferred to cold 0.25 M sucrose solution. These tissues were homogenized using "Polytron" homogenizer at top speed for 4 seconds. The homogenizer was connected to a timer so that the time could be kept constant for each homogenization. The homogenate was centrifuged in polythene centrifuge tubes for one hour at 100,000 g using a Spinco 40 or 65 rotor in a Beckman ultra centrifuge (Model L2-65B). The supernatant was decanted and the pellet suspended in cold 0.25 M sucrose to give a 10% to 15% homogenate (W/V). This is designated throughout this thesis as a pellet homogenate (PH) the pH of this fraction was 6.8. The plasma membrane and other subcellular fractions were prepared using the method of Kidwai et al (1971) with slight modification as follows. The pellet homogenate (PH) (about 3.2 ml) was layered on a continuous sucrose density gradient prepared with 0.25 M sucrose and 2 M sucrose



using an "ISCO" gradient former (Model 570). These gradient tubes were centrifuged at 30,000 rpm (111,688 g) for 2 hours using a Spinco SW 40 rotor. The four different layers namely of plasma membrane ( $F_1$ ), endoplasmic reticulum ( $F_2$ ), mitochondrial ( $F_3$ ) and of nuclear ( $F_4$ ) were aspirated into cold polythene centrifuge tubes and diluted with cold 0.25 M sucrose and centrifuged for 1 hour at 100,000 g. The pellets thus obtained were again suspended in the required volume of 0.25 M sucrose. In some instances the pellets were fixed using glutaraldehyde fixative and prepared for electronmicroscopy to be discussed later. Aliquots of different fractions were taken for counting radioactivity, protein determination and enzyme assays. Care was taken to carry out all these operations at 4°C or below.

#### Determination of Radioactivity

Aliquots of homogenate, PH, supernatant and other fractions were added to Bray's scintillator solution and the DPM were determined as described in Chapter III.

#### Protein Determination

Protein in different fractions were determined using the method of Lowry et al (1951) as modified by Miller (1959). A standard protein curve was prepared using a 1 mg/ml stock solution of bovine serum albumin.



### Measurement of 5'-Nucleotidase Activity

5'-Nucleotidase activity in the different fractions were measured using the method of Song and Bodansky (1967). AMP was used as the substrate. The  $P_i$  liberated was measured using the method of LeCocq and Inesi (1966).

### Measurement of $K^+$ -Stimulated p-nitrophenyl Phosphatase

$K^+$ -stimulated p-nitrophenyl phosphatase activity was measured in the presence of 50 mM imidazole buffer (pH 7.8), 5 mM  $MgSO_4$ , 10 mM KCl, 5 mM p-nitrophenyl phosphate (Na-salt) and incubated at 37°C for 15 to 20 minutes. The reaction was stopped by adding an equal volume of 10% TCA followed by the addition of twice the volume of 1 M Tris. The tubes were centrifuged and absorbance of the supernatant was measured at 400  $m\mu$  to estimate the amount of p-nitrophenol liberated. A standard curve for p-nitrophenol was prepared by using a stock solution of 1 mg/ml p-nitrophenol. The aliquots of the samples for standard curve were treated the same way as the enzyme reaction mixture.

### Cytochrome C-Oxidase Activity

This was measured using the method of Cooperstien and Lazarow (1959). The change in absorbance (i.e., rate of oxidation of reduced cytochrome c) per minute per mg protein was determined using a Gilford spectrophotometer (Model 2400). Cytochrome C was





reduced with (1.2 M) sodium hydrosulfite. The rate of oxidation of reduced cytochrome C was measured in different fractions, the rate being proportional to amount of enzyme present in each fraction. This enzyme activity was always determined within 8 hours after killing the animal.

### Electron Microscopy (EM)

The pellets of different fractions were fixed with phosphate buffered (pH 7.4) glutaraldehyde and kept overnight in the cold. Following the fixation, Mr. G. Duchon of this department prepared the pellets for EM as follows. The pellets were washed three to four times in Millonig's buffer, and post fixed with 1 ml of 1% osmium tetroxide solution for 1 hour. After water rinse, the pellets were dehydrated by passage through graded ethanol solutions and were finally washed in propylene oxide. Epon resin was used for embedding, and sections were cut with a diamond knife. They were then mounted on 200 mesh copper grids and after staining with lead citrate and uranyl acetate, were examined with a JEM-7A electron microscope.

### Measurement of Binding of Cardiac Glycosides to Isolated Plasma Membrane Fraction

Plasma membrane fraction containing 200 - 300  $\mu$ g protein/.2 ml was incubated for 10 minutes at 37°C in the presence of 50 mM histidine buffer at pH 7.4, with addition of 5 mM Mg ATP, 100 mM NaCl, 10 mM KCl





and  $^3\text{H}$ -ouabain or  $^3\text{H}$ -digitoxin ( $5 \times 10^{-8}$  M in the case of rabbits or  $10^{-4}$  M and  $5 \times 10^{-7}$  M in the case of rats) to make a total volume of 2 ml. The enzyme was preincubated for 10 minutes at  $37^\circ\text{C}$  in 10 ml polythene centrifuge tubes and the reaction started by adding Mg ATP solution containing radioactive glycoside. At the end of incubation it was centrifuged for 30 minutes at 100,000 g. The supernatant was discarded and the droplets wiped with Kleenex tissue and the pellet was suspended in 1 ml distilled water in the case of rat. Aliquots of this suspension were taken for counting radioactivity and for the determination of protein concentration. In the case of rabbit enzyme, the pellet was sometimes resuspended in cold distilled water and centrifuged for 30 minutes, and the pellet was finally suspended in 1 ml distilled water.

#### Solutions, Drugs and Chemicals

All the drugs and chemicals were dissolved in deionized water. The chemicals used and their sources are given below.

The substrates used were adenosine monophosphate (AMP), p-nitrophenyl phosphate (pNPP,  $\text{Na}^+$  salt) and adenosine-triphosphate (ATP,  $\text{Mg}^{++}$  salt). (All obtained from Sigma Chemicals). The pH of these substrates solutions were adjusted to 7 with either tris or histidine (both from Sigma).

Other chemicals used were NaCl, KCl,  $\text{MgSO}_4$  (Fisher),  $\text{NaHSO}_2$  (Fisher), sucrose (Fisher) and bovine serum albumin (Sigma).



## RESULTS

### Subcellular Fractionation

#### 1. Characterization of different fractions

5'-nucleotidase activity: Distribution of this enzyme in different fractions is shown in Table 15. Plasma membrane ( $F_1$ ) fraction contained the highest activity ( $19 \pm 1$   $\mu$ moles Pi/mg protein/hr). The activity is 4-fold greater than in PH ( $4.5 \pm 0.4$   $\mu$ moles Pi/mg protein/hr). There was some activity in endoplasmic reticulum fraction ( $F_2$ ), and mitochondrial fraction ( $F_3$ ) and nuclear fraction ( $F_4$ ).

$K^+$ -stimulated p-nitrophenyl phosphatase activity: The distribution of this enzyme in different fractions is also shown in Table 15.  $F_1$  was the only fraction which had the  $10^{-5}$  M ouabain or  $10^{-5}$  M digitoxin inhibited  $K^+$ -stimulated activity. The activity in  $F_1$  was  $1.8 \pm 0.3$   $\mu$ moles p-nitrophenol/mg protein/hr, which is 9 times higher than the activity in PH (0.22  $\mu$ moles p-nitrophenol/mg protein/hr). The activity in  $F_1$  was reduced to  $1.1 \pm 0.3$  by ouabain ( $10^{-5}$  M) and to 1.0 by digitoxin ( $10^{-5}$  M). The activity in PH was reduced to  $0.1 \pm 0.05$  by ouabain. Digitoxin did not have any effect.

Cytochrome oxidase activity: The activity is expressed as change in absorption/minute/mg protein. The results are shown in



TABLE 15

Enzyme activity in different fractions

	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>	F <sub>4</sub>	PH
5'-Nucleotidase <sup>†</sup>	(6) 19.0 ± 1	5.1 ± 0.5	2.6 ± 0.2	5.2 ± 0.5	4.5 ± 0.4
K <sup>†</sup> -activated pNPPase <sup>†</sup>	(6) 1.8 ± 0.3	0.35 ± 0.2	None	0.20 ± 0.1	0.22 ± 0.2
Ouabain (10 <sup>-5</sup> M) pNPPase <sup>†</sup>	(3) 1.1 ± 0.3	None	None	None	0.1 ± 0.05
Digitoxin (10 <sup>-5</sup> M) pNPPase <sup>†</sup>	(3) 1.0 ± 0.3	None	None	None	None
Cytochrome c-oxidase <sup>*</sup>	(3) 1.2 ± 0.2	3.0 ± 0.5	12.0 ± 2.0	0.5 ± 0.1	0.6 ± 0.1

<sup>†</sup> Specific activity expresses as  $\mu\text{moles Pi or PNP released/mg protein/hr} \pm \text{S.E.}$

<sup>\*</sup>  $\Delta\text{E/minute per mg protein.}$

Figures in parenthesis are number of experiments.

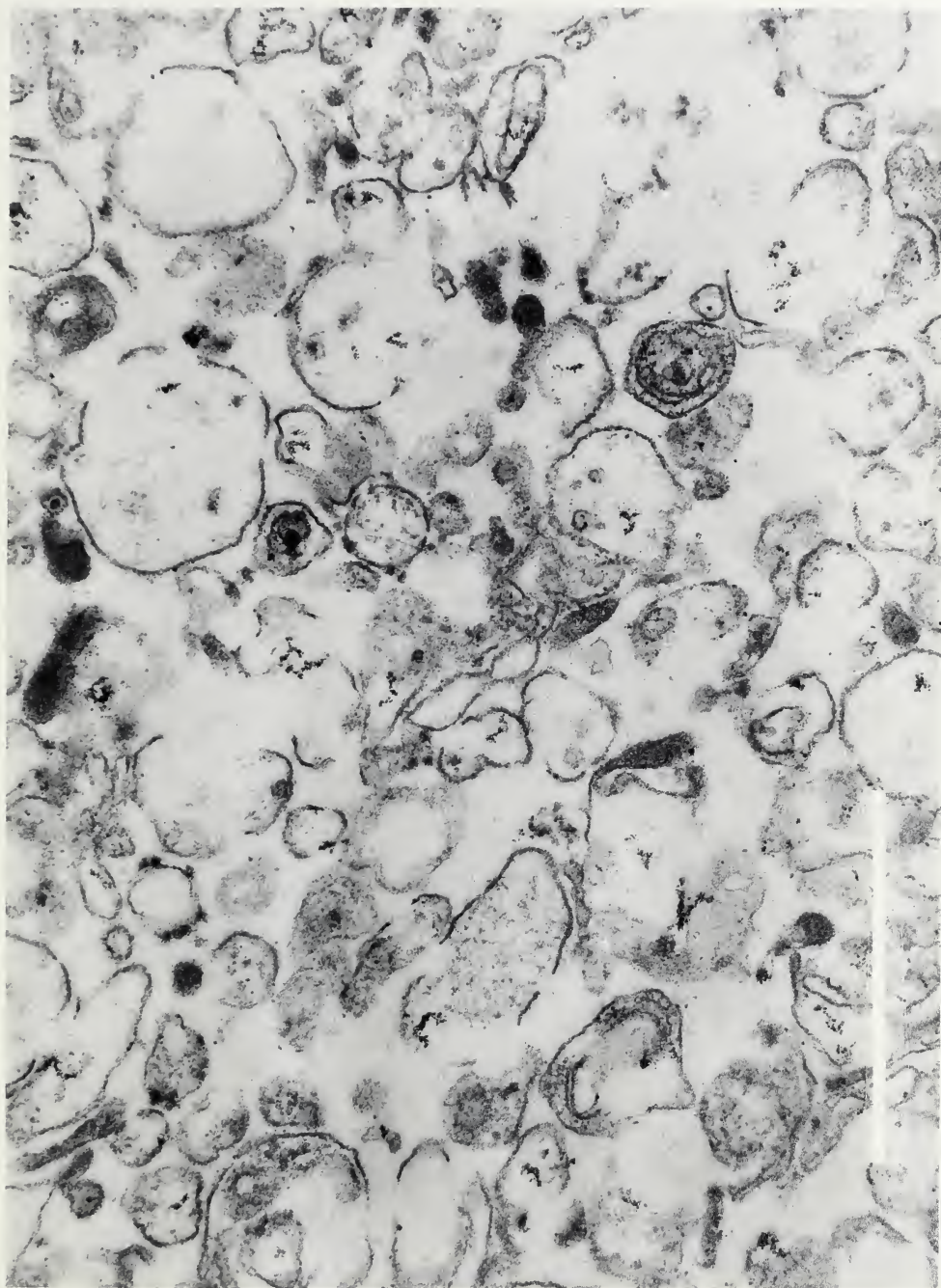


Table 15.  $F_3$  contained the highest activity ( $12 \pm 2$ ) which is 20 times the activity in PH ( $0.6 \pm .1$ ).  $F_1$ ,  $F_2$  and  $F_4$  had an activity of  $1.2 \pm 0.2$ ,  $3 \pm 0.5$  and  $0.5 \pm 0.1$  respectively.

Electron Microscopy: Electron microscopy of  $F_1$ ,  $F_2$ ,  $F_3$  and  $F_4$  is shown in plates 1, 2, 3 and 4 respectively.  $F_1$  consisted of open and closed vesicles and was found to be relatively free from mitochondrial contamination.  $F_2$  consisted of vesicles with dense granules probably the ribosomes and some smooth walled vesicles and occasional mitochondria.  $F_3$  was mostly mitochondria with few vesicles. Some mitochondria were broken and most of them were spherical.  $F_4$  consisted of nuclei, broken cells and mitochondria.

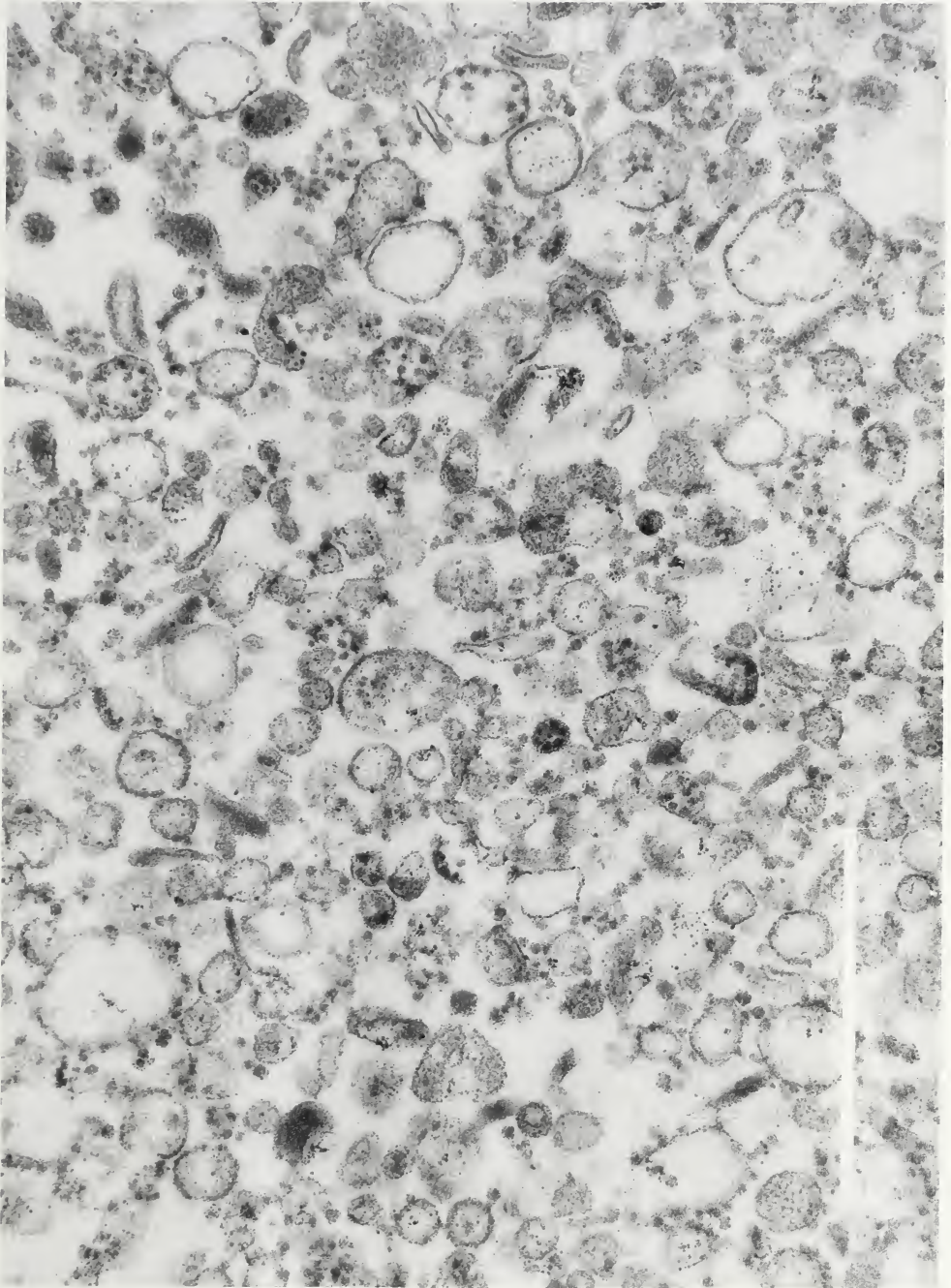






Electron micrograph of plasma membrane fraction ( $F_1$ ) x 25000.

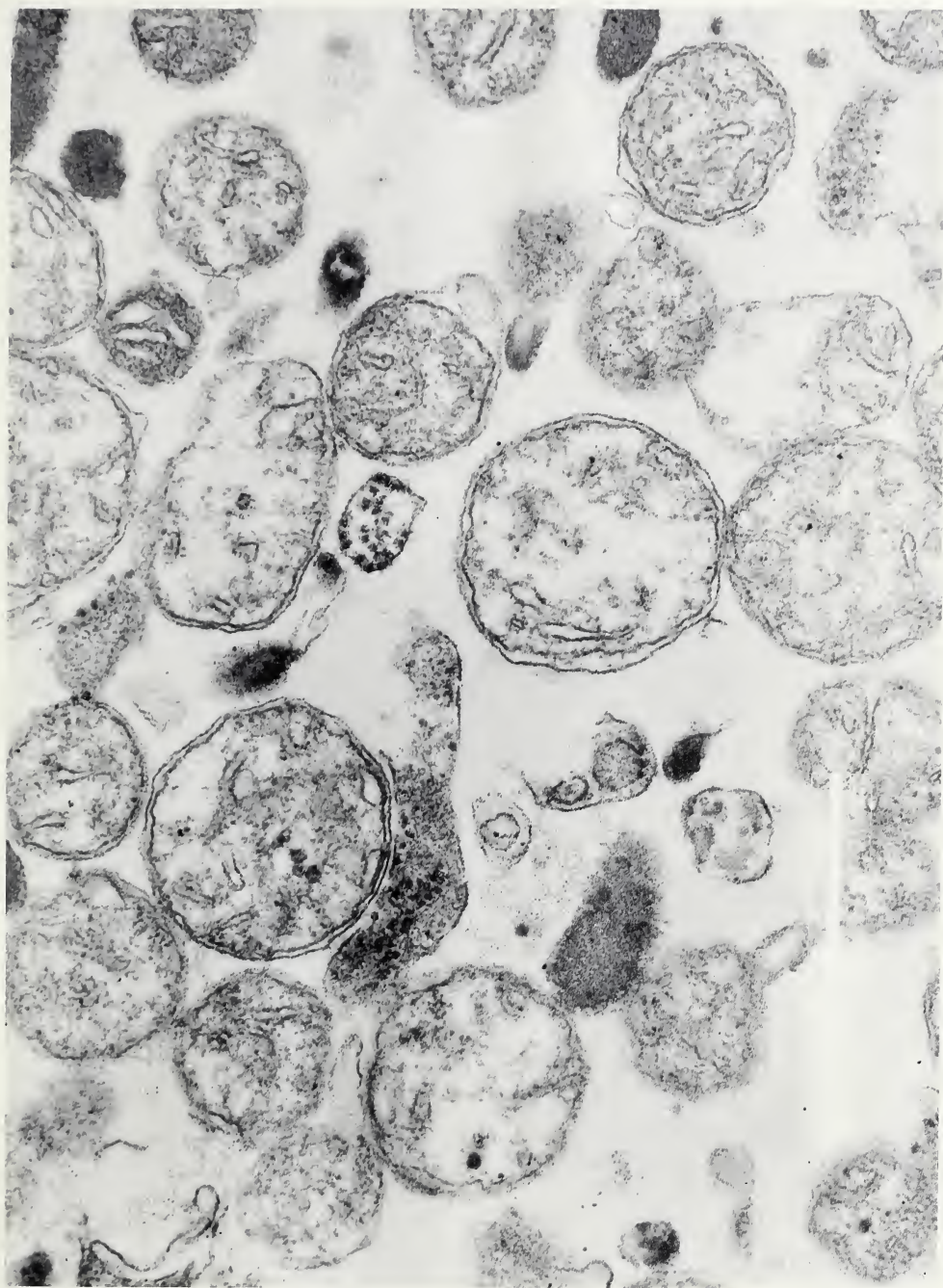




Electron micrograph of endoplasmic reticulum fraction ( $F_2$ )  $\times 25000$ .



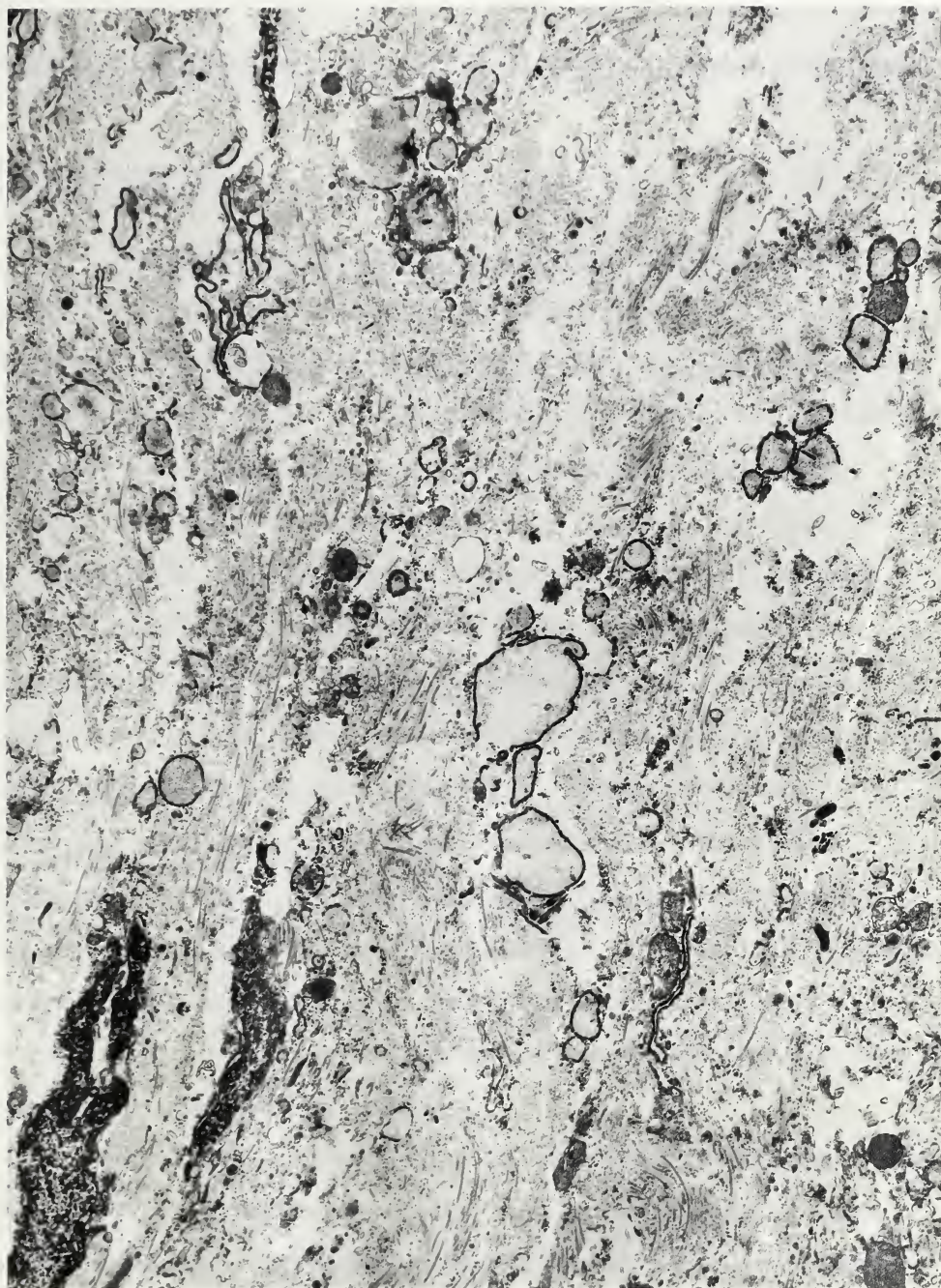




Electron micrograph of Mitochondrial fraction (F<sub>3</sub>) x 25000.







Electron micrograph of nuclear fraction ( $F_4$ )  $\times 25000$ .





SUBCELLULAR DISTRIBUTION OF  $5 \times 10^{-8}$  M  $^3\text{H}$ -OUABAIN IN RABBIT MYOMETRIUM

## Effect of Potassium

The binding of  $^3\text{H}$ -ouabain to different fractions is shown in Table 16. In NKR  $F_1$  bound about 0.58 pmoles/mg protein which was about four times higher than the binding to other fractions and twice the amount present in PH. In  $\text{K}^+$ -free Ringer the binding was increased by 5-fold, i.e., from 0.58 to 2.7 pmoles/mg protein. This amount was again four times the amount bound to  $F_2$ ,  $F_3$  and  $F_4$  and five times the amount in PH. The binding was increased not only in  $F_1$  but also in  $F_2$ ,  $F_3$  and  $F_4$ . This might probably be due to contamination of these fractions with  $F_1$ . In order to ensure that redistribution of ouabain did not occur during homogenization and isolation procedure control experiments were done in two ways. In one,  $10^{-5}$  M non-radioactive ouabain was added during homogenization. No decrease in the amount of bound  $^3\text{H}$ -ouabain occurred. In another experiment,  $^3\text{H}$ -ouabain was added to unlabelled homogenate. No appreciable binding occurred to different fractions. Thus the binding to different fractions seen in these experiments was not due to redistribution of  $^3\text{H}$ -ouabain during isolation procedure.

When 9.2 mM  $\text{K}^+$  Krebs Ringer was the uptake medium, the binding to  $F_1$  was only 0.22 pmoles/mg protein, only twice the amount present in other fractions. Increasing concentrations of  $\text{K}^+$  did not reduce the binding any further as shown in Table 16.



TABLE 16

Subcellular distribution of  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M)

Fractions	K <sup>+</sup> -free	N.K.R.	9.2 mM K <sup>+</sup>	23 mM K <sup>+</sup>	46 mM K <sup>+</sup>
F <sub>1</sub> (PM)	2.7*	0.58	0.23*	0.26*	0.27*
F <sub>2</sub> (ER)	0.7*	0.18	0.14	0.10	0.08*
F <sub>3</sub> (Mito)	0.34*	0.10	0.10	0.09	0.09
F <sub>4</sub> (Nuclear)	0.39*	0.15	0.09	0.08*	0.08*
PH	0.5*	0.32	0.14*	0.10*	0.12*
pmoles/mg protein					

\* Significant at 0.05 by Sheffe's test taking NKR as control.

S.E. is not included to avoid clutter.

N = 6.



### Effect of Sodium

In  $\text{Na}^+$ -free Ringer (choline  $\text{K}^+$ -free Ringer), the binding of ouabain to  $F_1$  was reduced to 0.42 pmoles/mg protein as compared to 1.8 pmoles/mg protein in  $\text{K}^+$ -free Ringer. When 9.2 mM  $\text{K}^+$  was present in  $\text{Na}^+$ -free Ringer the binding was reduced to 0.2 pmoles/mg protein, the same value as in Na containing medium. Apparently removal of Na inhibited ouabain binding to some of the sites at which  $\text{K}^+$  prevented the binding. These results are shown in Table 17.

### Effect of $10^{-5}$ M Digitoxin

Digitoxin reduced the binding of  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain to 0.1 pmoles/mg protein from 2.0 pmoles, which is less than the residual binding in the presence of 46 mM  $\text{K}^+$  alone (which is 0.29 pmoles/mg protein). In order to see if this ouabain binding inhibited by  $\text{K}^+$  was the same as that inhibited by digitoxin, the glycoside was added in the presence of 46 mM  $\text{K}^+$ . Again the binding was lowered to 0.1 pmoles/mg protein from 0.29 pmoles/mg protein. Therefore digitoxin prevented ouabain binding at the same sites as  $\text{K}^+$ , but affected other sites as well. These results are shown in Table 18.

### Effect of $10^{-7}$ M Non-radioactive Ouabain and $10^{-7}$ M Digitoxin

$10^{-7}$  M ouabain reduced the binding of  $5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain to  $F_1$  from 2.7 pmoles to 1.1 pmoles/mg protein.  $10^{-7}$  M digitoxin reduced it to 2 pmoles/mg protein.



TABLE 17

Effect of Na on distribution of  $^3\text{H}$ -ouabain.

Fractions	Na - KR		Na-free KR	
	$\text{K}^+$ -free	9.2 mM $\text{K}^+$	Choline $\text{K}^+$ -free	Choline + 9.2 mM $\text{K}^+$
$\text{F}_1$	1.8	0.23*	0.42	0.19*
$\text{F}_2$	0.5	0.09*	0.18	0.09*
$\text{F}_3$	0.2	0.08*	0.13	0.06*
$\text{F}_4$	0.2	0.05*	0.05	0.05*
pH	0.36	0.08*	0.10	0.06
pmoles/mg protein				

Values are means of at least 5 experiments.

\* Significantly different from control values ( $p < .05$ ) by Sheffe's test.

S.E. not included to avoid clutter.

N = 5.





TABLE 18

Effect of  $10^{-5}$  M digitoxin on the subcellular distribution of  $^3\text{H}$ -ouabain  
( $5 \times 10^{-8}$  M)

Fractions	$\text{K}^+$ -free	+ Digitoxin	46 mM $\text{K}^+$	46 mM $\text{K}^+$ + Digitoxin
$\text{F}_1$	2.5	0.1*	0.29	0.12*
$\text{F}_2$	0.8	0.06	0.09	0.07
$\text{F}_3$	0.3	0.05	0.04	0.05
$\text{F}_4$	0.4	0.04	0.04	0.04
PH	0.6	0.08	0.12	0.09
pmoles/mg protein				

\* Significantly different from controls ( $p < .05$ ) by Sheffe's test.

S.E. not included to avoid clutter.

N = 5.



## SUBCELLULAR DISTRIBUTION OF $^3\text{H}$ -DIGITOXIN ( $5 \times 10^{-8}$ M) IN RABBIT MYOMETRIUM

### Effect of Potassium

The results are shown in Table 19. In NKR the binding to  $F_1$  was about 0.5 pmoles/mg protein. Other fractions and PH bound about 0.14 pmoles and 0.5 pmoles respectively. The binding to  $F_1$  was about five times higher than to  $F_2$ ,  $F_3$  and  $F_4$ . Control experiments as with  $^3\text{H}$ -ouabain, showed that the binding was not due to redistribution. In  $\text{K}^+$ -free Ringer this binding did not increase in contrast to the results with  $^3\text{H}$ -ouabain. It was still about 0.58 pmoles/mg protein. 9.2 mM  $\text{K}^+$  also had very little effect on the binding. However, 13.8 mM  $\text{K}^+$  and higher concentrations of  $\text{K}^+$  reduced the binding of digitoxin to  $F_1$  and PH. Digitoxin binding to other fractions was little affected. 23 mM  $\text{K}^+$  reduced the binding to  $F_1$  maximally about half to about 0.24 pmoles/mg protein.

### Effect of Sodium

This is shown in Table 20. In  $\text{Na}^+$ -free Ringer the binding to  $F_1$  was reduced to 0.40 pmoles/mg protein from 0.65 pmoles/mg protein and addition of 23 mM  $\text{K}^+$  reduced this binding further to 0.20 pmoles/mg protein whether or not Na was present. As with ouabain binding, removal of Na seemed to inhibit digitoxin binding to some of the sites sensitive to high  $\text{K}^+$  concentration.



TABLE 19

Effect of  $K^+$  on the subcellular distribution of  $^3H$ -digitoxin ( $5 \times 10^{-8}$  M)

Fraction	$K^+$ -free	N.K.R.	9.2 mM $K^+$	13.8 mM $K^+$	23 mM	46 mM
$F_1$	0.58	0.50	0.45	0.29*	0.25*	0.25*
$F_2$	0.14	0.13	0.14	0.10	0.11	0.10
$F_3$	1.11	0.11	0.12	0.09	0.10	0.10
$F_4$	0.12	0.10	0.08	0.08	0.09	0.12
PH	0.55	0.50	0.46	0.30	0.28	0.29

pmoles/mg protein

\* Significantly different from values of N.K.R. by Sheffe's test ( $p < .05$ ).

S.E. not included to avoid clutter.

N = 5.



TABLE 20

Effect of Na on the subcellular distribution of  $^3\text{H}$ -digitoxin ( $5 \times 10^{-8}$  M)

Fractions	K <sup>+</sup> -free	+ 23 mM K <sup>+</sup>	Choline K <sup>+</sup> -free <sup>a</sup>	Choline + 23 mM K <sup>+</sup> <sup>a</sup>
F <sub>1</sub>	0.65	0.22 <sup>*</sup>	0.40 <sup>†</sup>	0.20 <sup>*</sup>
F <sub>2</sub>	0.20	0.10	0.12	0.10
F <sub>3</sub>	0.21	0.14	0.18	0.09
F <sub>4</sub>	0.19	0.15	0.15	0.12
PH	0.40	0.20	0.22	0.18
pmoles/mg protein				

\* Significantly different from controls ( $p < .05$ ).

† Significantly different from K<sup>+</sup>-free ( $p < .05$ ).

S.E. not included to avoid clutter.

N = 5.

<sup>a</sup> NaCl in the medium was replaced by equimolar concentration of choline-chloride.





### Effect of $10^{-5}$ M Ouabain

$10^{-5}$  M ouabain reduced the binding to  $F_1$  to 0.1 pmoles/mg protein, again a value lower than with potassium alone. In the presence of 46 mM  $K^+$ , ouabain reduced the binding to  $F_1$  to 0.09 pmoles, a similar value. These results suggest that ouabain inhibited digitoxin binding to the sites sensitive to K and also to additional sites. These results are shown in Table 21.

### BINDING OF $^3H$ -OUABAIN ( $5 \times 10^{-8}$ M) TO PREVIOUSLY ISOLATED PLASMA MEMBRANE FRACTION (PM)

#### Effect of Washing with Cold Distilled Water on Binding

This is shown in Table 22. About  $3.9 \pm 0.05$  pmoles/mg protein of ouabain was associated with PM after the initial centrifugation. On washing once by resuspending the pellet in cold water, the amount bound was reduced to  $3.0 \pm 0.2$  pmoles/mg protein. On second wash the binding was reduced to  $2.7 \pm 0.15$  pmoles/mg protein. Thus two washings eliminated about 1.2 pmoles per/mg protein of the loosely bound glycoside.

#### Characteristics of $^3H$ -Ouabain Binding

These results are shown in Table 23. The binding in the presence of Mg ATP was 1.0 pmoles/mg protein, and this was increased by Na to 3.0 pmoles. In the presence of tris ATP the binding was



TABLE 21

Effect of  $10^{-5}$  M ouabain on  $^3\text{H}$ -digitoxin distribution

Fractions	$\text{K}^+$ -free	$\text{K}^+$ -free + Ouabain	46 mM $\text{K}^+$	46 mM $\text{K}^+$ + Ouabain
$\text{F}_1$	0.61	0.10*	0.25	0.09*
$\text{F}_2$	0.16	0.08*	0.15	0.07*
$\text{F}_3$	0.13	0.09*	0.14	0.06*
$\text{F}_4$	0.15	0.09*	0.15	0.05*
PH	0.58	0.20*	0.24	0.22
pmoles/mg protein				

\* Significantly different from control ( $p < .05$ ) by Sheffe's test.

S.E. not included to avoid clutter.

N = 4.



TABLE 22

Effect of washing on binding of cardiac glycosides to isolate plasma membrane from rabbit myometrium

Washings	Cardiac Glycosides	
	Ouabain	Digitoxin
No wash	$3.9 \pm 0.5$	$3.7 \pm 0.8$
One wash	$3.0 \pm 0.2$	$1.2 \pm 0.4$
Two washes	$2.7 \pm 0.15$	$0.90 \pm 0.2$
pmoles/mg protein $\pm$ S.E.		

N = 4.



TABLE 23

$5 \times 10^{-8}$  M  $^3\text{H}$ -ouabain binding to rabbit myometrial plasma membrane

Additions	Total	Specific
1. $\text{Na}^+$ + Mg ATP	3.0 $\pm$ 0.3	2.68
2. Mg ATP	1.0* $\pm$ 0.1	0.68
3. Tris ATP	0.9* $\pm$ 0.08	0.58
4. Tris ATP + 2 mM EDTA	0.56* $\pm$ 0.05	0.11
5. $\text{Na}^+$ + Mg ATP + 5 mM $\text{K}^+$	0.95* $\pm$ 0.02	0.63
6. $\text{Na}^+$ + Mg ATP + 10 mM $\text{K}^+$	0.67* $\pm$ 0.03	0.35
7. $\text{Na}^+$ + Mg ATP + 20 mM $\text{K}^+$	0.55* $\pm$ 0.02	0.23
8. $\text{Na}^+$ + Mg ATP + Ouabain $10^{-5}$ M	0.32* $\pm$ 0.04	
9. $\text{Na}^+$ + Digitoxin $10^{-5}$ M	0.50* $\pm$ 0.06	0.18
10. None	0.45* $\pm$ 0.08	0.13
pmoles/mg protein $\pm$ S.E.		

\* Significantly different from No. 1 by Sheffe's test ( $p < .01$ ).

N = 12.





0.9 pmoles. Addition of 2 mM EDTA reduced the binding to 0.56 pmoles, a value not significantly different from the non-specific binding, i.e., the binding in the presence of  $10^{-5}$  M non-radioactive ouabain. Thus, there was probably some  $Mg^{++}$  in the plasma membrane fraction to support some specific binding. The non-specific binding for  $^3H$ -ouabain was  $0.32 \pm 0.04$  pmoles/mg protein. The specific binding occurring in different media is expressed as the difference between total binding and non-specific binding. Thus specific binding in the presence of  $Na^+$ ,  $Mg^{++}$  ATP was 2.68 pmoles and with  $Mg^{++}$  ATP 0.68 pmoles; with tris ATP, 0.58 pmoles and with tris ATP and EDTA 0.11 pmoles/mg protein. 5 mM  $K^+$  reduced the specific binding to 0.63 from 2.68 and 10 mM  $K^+$  reduced it further to 0.35. 20 mM  $K^+$  reduced the specific binding to 0.23, probably not significantly different from zero. When  $Mg^{++}$  ATP was present the specific binding was only 0.12 pmoles. These values of Na-dependent and  $K^+$ -sensitive binding of ouabain to isolated PM are thus very similar to the ones obtained in subcellular fractionation experiments (see Tables 16 and 17).

#### BINDING OF $5 \times 10^{-8}$ M $^3H$ -DIGITOXIN TO ISOLATED PLASMA MEMBRANE OF RABBIT MYOMETRIUM

##### Effect of Washing

This is shown in Table 22. The value after first centrifugation was 3.7 pmoles/mg protein, a value much higher than obtained in



subcellular fractionation experiments. Two washings were sufficient to get rid of most of the loosely bound digitoxin, about 2.8 pmoles/mg protein. 0.9 pmoles/mg protein remained bound.

### Binding Characteristics

This is shown in Table 24. Specific binding in the presence of Mg ATP was 0.45 pmoles/mg protein. Addition of  $\text{Na}^+$  did not change this binding and was 0.30 pmoles /mg protein. 20 mM  $\text{K}^+$  reduced the binding to 0.1 pmoles/mg protein, but the reduction was not significant by Sheffe's test ( $p > 0.5$ ). In the presence of  $\text{MgCl}_2$  alone the binding was 0.55 and 20 mM  $\text{K}^+$  reduced it to 0.35 pmoles/mg protein, a very insignificant effect. Where no  $\text{Mg}^{++}$  or Mg ATP was present no detectable specific binding occurred.

### Binding of $2.5 \times 10^{-7}$ M $^3\text{H}$ -Digitoxin

This concentration was used to see if any specific binding could be detected. The rationale was in the intact tissue, the intracellular concentration of digitoxin is 5 times the extracellular concentration and this might have been responsible for the detection of  $\text{Na}^+$ -dependent binding in subcellular fractionation experiments. These results are shown in Table 25. The specific binding in the presence of Na and Mg ATP was 2.4 pmoles and with Mg ATP it was 2.0 pmoles/mg protein. 20 mM  $\text{K}^+$  reduced this binding to 1.5 pmoles/mg protein. In the absence of Mg ATP the specific



TABLE 24

$5 \times 10^{-8}$  M  $^3\text{H}$ -digitoxin binding to rabbit myometrial plasma membrane

Additions	Total	Specific
1. $\text{Na}^+ + \text{Mg ATP}$	$0.75 \pm 0.1$	0.30
2. $\text{Mg ATP}$	$0.90 \pm 0.09$	0.45
3. $\text{Na} + \text{Mg ATP} + 20 \text{ mM K}^+$	$0.55 \pm 0.09$	0.10
4. None	$0.45 \pm 0.08$	
5. $\text{Na} + \text{Mg ATP} + 10^{-5} \text{ M digitoxin}$	$0.45 \pm 0.10$	
6. $\text{MgCl}_2$	$1.0 \pm 0.13$	0.55
7. $\text{MgCl}_2 + 20 \text{ mM K}^+$	$0.80 \pm 0.08$	0.35
pmoles/mg protein $\pm$ S.E.		

N = 5.



TABLE 25

$2.5 \times 10^{-7}$  M  $^3\text{H}$ -digitoxin binding to rabbit myometrial plasma membrane

Additions	Total	Specific
1. Na + Mg ATP	4.0 $\pm$ 0.31	2.4
2. Mg ATP	3.6 $\pm$ 0.08	2.0
3. Na + Mg ATP + 20 mM K <sup>+</sup>	3.1 $\pm$ 0.06	1.5
4. None	2.0* $\pm$ 0.08	0.4
5. Na + Mg ATP + $10^{-5}$ M Digitoxin	1.5* $\pm$ 0.10	-
6. MgCl <sub>2</sub>	4.2 $\pm$ 0.40	2.7
pmoles/mg protein $\pm$ S.E.		

\* Significantly different from No. 1 by Sheffe's test ( $p < .05$ ).

N = 5.





binding was 0.4 pmoles. In the presence of  $Mg^{++}$  alone the binding was 2.7 pmoles. These results suggest that digitoxin has a lower affinity than ouabain for  $Na^{+}-K^{+}$ -ATPase and the results obtained with intact tissue might be due to the higher intracellular concentration of digitoxin.

#### SUBCELLULAR DISTRIBUTION OF $^3H$ -OUABAIN IN RAT MYOMETRIUM

##### Subcellular Distribution of $10^{-6}$ M $^3H$ -Ouabain in Rat Myometrium

This is shown in Table 26. In NKR  $F_1$  bound 0.71 pmoles/mg protein. PH contained 0.65 pmoles/mg protein. The ouabain binding to  $F_1$  was twice that in other fractions. In  $K^{+}$ -free Krebs Ringer the binding to  $F_1$  or other fractions did not go up. 46 mM  $K^{+}$  also had no effect on this binding. Control experiments showed that no redistribution occurred during isolation procedure.

##### Subcellular Distribution of $5 \times 10^{-8}$ M $^3H$ -Ouabain in Rat Myometrium

This is shown in Table 27.  $F_1$  in NKR bound 0.11 pmoles/mg protein and other fractions and PH bound about 0.08 pmoles/mg protein. Binding to  $F_1$  in  $K^{+}$ -free Ringer did not go up.



TABLE 26

Subcellular distribution of  $^3\text{H}$ -ouabain  $10^{-6}$  M in rat myometrium

Fractions	$\text{K}^+$ -free	N.K.R.	46 mM $\text{K}^+$
$\text{F}_1$	$0.65 \pm 0.12$	$0.71 \pm 0.15$	$0.64 \pm 0.11$
$\text{F}_2$	$0.25 \pm 0.05$	$0.4 \pm 0.03$	$0.30 \pm 0.06$
$\text{F}_3$	$0.35 \pm 0.06$	$0.39 \pm 0.04$	$0.32 \pm 0.07$
$\text{F}_4$	$0.15 \pm 0.02$	$0.15 \pm 0.02$	$0.14 \pm 0.02$
PH	$0.60 \pm 0.10$	$0.65 \pm 0.09$	$0.63 \pm 0.12$
pmoles/mg protein $\pm$ S.E.			

N = 4.



TABLE 27

Subcellular distribution of  $^3\text{H}$ -ouabain  $5 \times 10^{-8}$  M in rat myometrium

Fractions	$\text{K}^+$ -free	N.K.R.
$\text{F}_1$	$0.12 \pm 0.03$	$0.115 \pm 0.02$
$\text{F}_2$	$0.10 \pm 0.04$	$0.09 \pm 0.03$
$\text{F}_3$	$0.07 \pm 0.02$	$0.08 \pm 0.02$
$\text{F}_4$	$0.05 \pm 0.01$	$0.07 \pm 0.02$
PH	$0.10 \pm 0.04$	$0.08 \pm 0.03$
pmoles/mg protein $\pm$ S.E.		



## Binding of $10^{-4}$ M and $5 \times 10^{-7}$ M $^3\text{H}$ -Ouabain to Isolated Rat Myometrial Plasma Membrane

These results are shown in Table 28. With either concentrations no Na-dependent, ATP-dependent or  $\text{K}^+$ -inhibited binding could be detected. Unlabelled ouabain also had very little effect on this binding.

### Effect of Washing

$10^{-4}$  M  $^3\text{H}$ -ouabain was bound to isolated rat myometrial pm and washed twice with cold water after first centrifugation. Most of the ouabain was washed off by this procedure. The binding reduced from 6000 pmoles to 10 pmoles/mg protein. But no Na or ATP-dependent binding could be detected.  $\text{K}^+$  also had no effect on this binding. (Data not shown).





TABLE 28

Binding of  $^3\text{H}$ -ouabain to isolated plasma membrane from rat myometrium

Additions	$5 \times 10^{-7}$ M	$10^{-4}$ M
$\text{Na}^+ + \text{Mg ATP}$	$0.5 \pm 0.10$	$5900 \pm 150$
$\text{Na}^+ + \text{Mg ATP} + 10 \text{ mM } \text{K}^+$	$0.52 \pm 0.11$	$5900 \pm 120$
Mg ATP	$0.48 \pm 0.08$	$6800 \pm 200$
$\text{MgCl}_2$	$0.50 \pm 0.09$	$6200 \pm 180$
$\text{Na Mg ATP} + 10^{-3} \text{ M Ouabain}$	$0.58 \pm 0.06$	$5500 \pm 165$
pmoles/mg protein $\pm$ S.E.		

N = 3.



## DISCUSSION

Before attempting to study the subcellular distribution of cardiac glycosides, we established that the fractions obtained by the method employed were reasonably pure. In rabbit myometrium, the distribution of marker enzymes showed that plasma membrane fraction ( $F_1$ ) (Table 15) was rich in 5'-nucleotidase and  $K^+$ -stimulated p-nitrophenyl phosphatase (pNPPase) and the mitochondrial fraction ( $F_3$ ) rich in cytochrome C-oxidase. The presence of 5'-nucleotidase in other fractions could be either due to the initial presence of this enzyme in the pure fractions or to the contamination with  $F_1$ . If there was contamination due to particles of  $F_1$  in other fractions, then the percentage contamination in  $F_2$  is 25% and in  $F_3$  13%. It is not certain that 5'-nucleotidase is present only in plasma membrane fraction (see Song et al, 1969). Ouabain inhibited,  $K^+$ -stimulated p-NPPase could be detected only in  $F_1$ .  $K^+$ -stimulated p-NPPase was also present in  $F_2$  and  $F_4$  but ouabain did not inhibit the  $K^+$ -stimulated enzyme. The failure to detect ouabain inhibition in these fractions could be due to the presence of very small amounts of  $K^+$ -stimulated p-NPPase, and the sensitivity of the method might have been a limitation. (The sensitivity limit was  $\pm 15\%$  of stimulation above the basal  $Mg^{++}$  phosphatase).

Mitochondrial contamination in  $F_1$  and other fractions on the basis of cytochrome C-oxidase activity was 10% in  $F_1$ , 25% in  $F_2$



and 2.5% in  $F_4$ . Thus on the basis of enzyme distribution the plasma membrane fraction was relatively pure. This was supported by the absence of mitochondrial materials in electron microscopic pictures of  $F_1$ .

In the case of rat myometrium, Kidwai et al (1971) have already shown that the plasma membrane fraction obtained by this method was relatively pure. Therefore, these studies were not repeated in the case of rat myometrium.

The results of subcellular distribution of  $^3\text{H}$ -ouabain and  $^3\text{H}$ -digitoxin in rabbit myometrium clearly suggest a difference in binding characteristics of these two glycosides. As plasma membrane is the site of interest for the present study, the discussion will be focussed on the binding of these glycosides to  $F_1$ . The subcellular distribution of  $^3\text{H}$ -ouabain was according to what one would expect if  $\text{Na}^+-\text{K}^+$ -ATPase was the main binding site.  $F_1$  contained the highest amount of  $^3\text{H}$ -ouabain (0.58 pmoles/mg protein) in experiments with NKR.  $F_2$ ,  $F_3$  and  $F_4$  bound about 0.16 pmoles/mg protein and PH bound about 0.25 pmoles/mg protein. The binding to  $F_1$  increased 5-fold in  $\text{K}^+$ -free Krebs and is favourable with the  $\text{K}^+$ -ouabain antagonism for binding to  $\text{Na}^+-\text{K}^+$ -ATPase as already discussed in the last chapter. The binding to  $F_2$ ,  $F_3$  and  $F_4$  also increased in  $\text{K}^+$ -free Krebs (about 2-fold) suggesting that these fractions were contaminated with  $F_1$  to some extent. On the basis of increase in binding in  $\text{K}^+$ -free media, the contamination in other



fractions by  $F_1$  would be 21% in  $F_2$  and 12% in  $F_3$  and  $F_4$ . This closely agrees with the distribution of 5'-nucleotidase.

The binding to  $F_1$  in  $K^+$ -free Krebs was 2.6 pmoles/mg protein which is similar to the value for ouabain binding to microsomal fraction from guinea pig hearts (Dutta and Marks, 1969). Present results also suggest that the binding of ouabain may be preferentially to the phosphorylated form of  $Na^+-K^+-ATPase$  as the binding was largely  $Na^+$ -dependent. It is known from literature that  $Na^+$  stimulates phosphorylation in the presence of  $Mg^{++}$  and ATP (Charnock et al, 1963 and Skou, 1960).

This binding was very sensitive to  $K^+$ , as 4.6 mM  $K^+$  reduced the binding from 2.7 to 0.58 pmoles/mg protein. Tobin and Sen (1970) have shown that ouabain has a very high affinity for the  $E_2 - P$  form of the enzyme but that  $K^+$  antagonizes the binding of ouabain to  $E_2 - P$  form. Present results agree with this suggestion. Similar binding of ouabain to  $E_2 - P$  form of  $Na^+-K^+-ATPase$  from different tissues have been shown by Akera and Brody (1971) and Allen et al (1971 a). The binding to  $F_1$ , in the present study, in the absence of  $Na^+$  and  $K^+$  was a little higher (but significant) than the  $K^+$ -inhibited binding. That is, omission of  $Na^+$  did not reduce the binding to the same extent as the action of  $K^+$  addition. The binding in the absence of  $Na^+$  and  $K^+$  was reduced by the addition of 9.2 mM  $K^+$ , suggesting that some  $K^+$ -sensitive binding occurred in  $Na^+$ -free media. Similar binding in the absence of  $Na^+$  was reported by Albers et al (1968) and Matsui and Schwartz (1967) for Electrophorus







electric organ and beef heart enzymes respectively. Post et al (1965) have reported that there is a certain amount of phosphorylation in the presence of  $Mg^{++}$  and ATP alone. Thus ouabain binding in the absence of  $Na^+$  may also be to the phosphorylated form of  $Na^+-K^+-ATPase$ .

There was about 10 meq/kg of  $Na^+$  still present in the tissue after 1 hour incubation in  $Na^+$ -free media (see Chapter I). This  $Na^+$  might have contributed to the formation of some phosphorylated enzyme.

The binding in the absence of  $Na^+$  might also be to the native enzyme or to the conformationally altered but non-phosphorylated  $E_2$  - form of the enzyme. However, binding to native enzyme has not been demonstrated to be antagonized by  $K^+$ . If the binding occurred to  $E_2$  (assuming this form can exist in the intact cell), then the binding to this form has been shown to be antagonized by  $K^+$  (see Tobin and Sen, 1970), and as the residual binding in the absence of  $Na^+$  was reduced by  $K^+$ , this possibility may not be ruled out.

$^3H$ -ouabain binding to  $F_1$  was reduced by  $10^{-5}$  M digitoxin and the reduction was greater than in high  $K^+$  media. This suggests that the  $K^+$ -sensitive ouabain binding site is competed for by other active cardiac glycosides. In addition, there appears to be competition at other sites. The extra reduction by digitoxin in the presence of high  $K^+$  is only about 4% of the total binding. Thus,  $K^+$  reduces most of the specific binding (Defined as the difference between the binding in  $K^+$ -free media and the binding in the presence of unlabelled glycosides). All these results suggested that ouabain binding to



$F_1$  involved  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and agrees with most of the recent reports on ouabain binding to different  $\text{Na}^+\text{-K}^+\text{-ATPase}$  preparations (Albers et al, 1968; Matsui and Schwartz, 1967 and Tobin and Sen, 1970).

In contrast to these results,  $^3\text{H}$ -digitoxin binding was not increased in  $\text{K}^+$ -free Krebs. 9.2 mM  $\text{K}^+$  also had very little effect on  $^3\text{H}$ -digitoxin binding to  $F_1$ . 23 mM  $\text{K}^+$  and higher concentrations of  $\text{K}^+$  reduced the binding to 0.2 pmoles/mg protein from 0.6 pmoles/mg protein, a level similar to ouabain binding in the presence of 9.2 mM  $\text{K}^+$ . This suggested that digitoxin binding was relatively insensitive to  $\text{K}^+$  as compared to ouabain binding. Also while ouabain binding was as high as 2.7 pmoles/mg protein in  $\text{K}^+$ -free media, digitoxin binding never exceeded 0.68 pmoles/mg protein. This suggests that ouabain has a higher affinity than digitoxin to  $\text{E}_2 - \text{P}$  form of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . This is surprising because digitoxin is 5 times more effective than ouabain in inhibiting  $\text{Na}^+\text{-K}^+\text{-ATPase}$  from guinea pig brush border (Leopold et al, 1971). This would suggest then that binding and inhibition may not be related. On the other hand, the conditions under which the enzyme inhibition was studied contained  $\text{K}^+$  and ouabain binding is more sensitive to  $\text{K}^+$  than digitoxin binding. This might have resulted in a greater inhibition with digitoxin than ouabain. There are very few enzyme studies where digitoxin and ouabain effects are compared using the same enzyme preparation. It is therefore uncertain if digitoxin is more potent than ouabain in inhibiting  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . The differences could also be due to different species and tissues used.



In  $\text{Na}^+$ -free and  $\text{K}^+$ -free media the binding of  $^3\text{H}$ -digitoxin was reduced to 0.4 pmoles/mg protein from 0.68 picomoles/mg protein. The difference though slight, was significant. The binding was reduced further by 23 mM  $\text{K}^+$  to 0.2 pmoles/mg protein. The  $\text{Na}^+$ -dependent binding of digitoxin suggests that a part of digitoxin binding involved a phosphorylated intermediate of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , but it is difficult to explain the insensitivity of this binding to  $\text{K}^+$ .

One possibility is that digitoxin binds to  $\text{E}_1 - \text{P}$  form of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  rather than to the  $\text{E}_2 - \text{P}$  form. If we consider the following scheme of ATPase phosphorylation (Albers et al, 1968 and Post et al, 1969):



$\text{E}_1$  is the native enzyme,  $\text{E}_1 - \text{P}$  is the phosphorylated form, requiring  $\text{Na}^+$  for its formation and  $\text{E}_2 - \text{P}$  is the  $\text{K}^+$  sensitive form of phosphoenzyme. It was seen in the last chapter that digitoxin concentration inside the cell was about 5 times higher than its extracellular concentration (Calculated from a S/M ratio of 3 ml/g and inulin space of 0.45 ml/g). Therefore, the  $\text{E}_1 - \text{P}$  form which is intracellularly oriented may be exposed to a 5 times higher concentration of digitoxin than ouabain when tissues are exposed to equal concentrations of the two glycosides. Furthermore, its lipid solubility may cause digitoxin to be concentrated in the phospholipid layers of the plasma membrane. Ouabain has a very low affinity for  $\text{E}_1 - \text{P}$  (Tobin and Sen, 1970). If such differences between the two glycosides resulted in digitoxin being bound more than





ouabain to  $E_1 - P$ ,  $K^+$  would have less effect on its binding as  $K^+$  has a lower affinity for this form (Tobin and Sen, 1970). This could explain the relative insensitivity of digitoxin binding to  $K^+$ . The effect of 23 mM  $K^+$  and higher concentrations of  $K^+$  may be due to inhibition of formation of any phosphorylated intermediate by this ion (Skou, 1960). It is also possible that digitoxin binds to non-phosphorylated enzyme and  $Na^+$  effect may be allosteric.

As the differences between  $^3H$ -digitoxin and  $^3H$ -ouabain binding to  $F_1$  could have been due to differences in transmembrane distribution of these drugs, studies on binding of these two glycosides to isolated plasma membrane fraction (pm) were carried out. Such a procedure would provide a similar milieu for binding of both the glycosides. Initial experiments with  $5 \times 10^{-8}$  M  $^3H$ -digitoxin binding to pm gave a higher binding than the binding in subcellular distribution experiments. Most of this binding was non-specific as unlabelled digitoxin did not reduce the binding very much. In the subcellular fractionation experiments, before the pellet of  $F_1$  was obtained, it was subjected to two washings, i.e., resuspension of PH and resuspension of  $F_1$  layer from sucrose density gradient. This might have resulted in the loss of most of the non-specific binding. When the pellets obtained after first centrifugation, in the case of isolated pm experiments (see Results) were washed by resuspending twice in cold water most of the loosely bound  $^3H$ -digitoxin (70%) was washed off. In the case of  $^3H$ -ouabain, much less of pm bound





$^3\text{H}$ -ouabain was released by this procedure (only 23% was released).

Thus in contrast to digitoxin, most of the ouabain binding was specific.

The binding of  $^3\text{H}$ -ouabain ( $5 \times 10^{-8}$  M) to isolated pm was  $\text{Mg}^{++}$  and ATP-dependent and was increased by  $\text{Na}^+$ , reduced by 5 mM  $\text{K}^+$ , unlabelled ouabain ( $10^{-5}$  M) or digitoxin ( $10^{-5}$  M). Also the amount bound in the presence of  $\text{Mg}^{++}$ ,  $\text{Na}^+$  and ATP was 2.7 pmoles/mg protein, a value very similar to the value obtained in subcellular fractionation experiments. These results again suggested that ouabain bound to  $\text{E}_2 - \text{P}$  form of  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  as reported by Tobin and Sen (1970).

In the case of  $^3\text{H}$ -digitoxin, the results of binding experiments are more complicated. With  $5 \times 10^{-8}$  M digitoxin, the binding was not significantly affected by omission of  $\text{Na}^+$  or ATP and  $\text{K}^+$  reduced this binding only slightly.  $\text{Mg}^{++}$  alone could support the specific binding. Possibly, the intracellular concentration of digitoxin in the fractionation experiments was 5 times higher than the extracellular, allowing detection of  $\text{Na}^+$ -dependent binding as there was a higher concentration of glycoside available to bind to the enzyme from inside. To test this possibility, binding of  $2.5 \times 10^{-7}$  M  $^3\text{H}$ -digitoxin was carried out. With this concentration the non-specific binding (that not reduced by unlabelled digitoxin) increased 4-fold. Again  $\text{Na}^+$  omission had insignificant effect on specific binding and 20 mM  $\text{K}^+$  also failed to reduce it significantly. In the absence of  $\text{Mg}^{++}$  and ATP, the binding of digitoxin was reduced



to the level of non-specific binding.  $Mg^{++}$  alone could support the specific binding. This suggested that for digitoxin binding to  $Na^+-K^+-ATPase$  phosphorylated form was not essential, but  $Mg^{++}$  in some way alters the enzyme so that digitoxin can bind to it. It is possible that the results obtained in subcellular fractionation experiments could not be obtained in the isolated system because of the absence of  $E_1 - P$  in the isolated system under the conditions studied, i.e., the presence of 5 mM  $Mg^{++}$  converts all  $E_1 - P$  to  $E_2 - P$  rapidly and irreversibly (Post et al, 1969). To stabilize  $E_1 - P$  in the isolated system, treatment with oligomycin or N-ethyl maleimide was necessary. As we did not want to introduce another variable, this procedure was not tried. Reducing  $Mg^{++}$  concentration is another way of preventing the conversion of  $E_1 - P$  to  $E_2 - P$ . The experiments where the existence of  $E_1 - P$  is shown are mostly carried out at  $0^\circ C$  using short term incubations (Post et al, 1969). Such conditions are not suitable for binding studies (as cardiac glycoside binding is temperature dependent).

Binding of  $^3H$ -digitoxin to  $E_2 - P$  has to be ruled out because of the lack of  $K^+$  effect.  $K^+$  has a very high affinity for  $E_2 - P$  and any binding of digitoxin to  $E_2 - P$  would be sensitive to  $K^+$ . Though digitoxin binds to a form other than  $E_2 - P$ , it probably binds to the same site as ouabain site, because the two glycosides can displace each other from their binding site.

These results, therefore, suggest that ouabain binding is highly specific requiring  $Mg^{++}$ , ATP and  $Na^+$  while digitoxin binding



is not. Also these two glycosides have different binding affinities for the two different forms of the  $\text{Na}^+-\text{K}^+$ -ATPase in rabbit myometrial pm.

In the case of rat myometrial pm no specific binding of ouabain could be detected in subcellular fractionation experiments, with either  $10^{-4}$  M or  $10^{-6}$  M  $^3\text{H}$ -ouabain. There are three possible explanations for this result.

1) The ouabain enzyme complex may be very labile and it dissociated completely during the isolation procedure. Such a rapid dissociation of ouabain enzyme complex for rat heart enzyme was shown by Allen and Schwartz (1969). The control experiments showed that there was no redistribution occurring during isolation procedure. But this does not rule out the rapid dissociation, because if the enzyme ouabain complex dissociated during homogenization and no reuptake by different fractions occurred, the dissociation would go undetected. When, however, binding to isolated pm was carried out to prevent such a dissociation, still the specific binding was not detectable.

2. The specific binding may be very little and not detectable by the present method because of very high non-specific binding. To test this possibility the pellet after binding  $10^{-4}$  M  $^3\text{H}$ -ouabain was washed twice to get rid of non-specific uptake. This procedure also failed to show the specific binding.





3. No specific binding may be occurring to rat myometrial pm. This possibility raises many problems such as how can one explain the ouabain inhibition of ion movements in rat myometrium (Daniel, 1964 a,b), and the  $K^+$  antagonism of enzyme inhibition by ouabain. It is reported by Akera et al (1969) and Allen and Schwartz (1969) and many others that rat heart enzyme requires higher concentration of ouabain for inhibition but  $K^+$  antagonism is the same as that for the enzymes from the sensitive species. Allen and Schwartz (1969) showed that rat heart enzyme-ouabain complex dissociated immediately on dilution as estimated by the reversibility of enzyme inhibition. They have measured neither the extent of binding before and after washing, nor the effect of  $K^+$  on such binding. Leopold et al (1971) have reported that there is no correlation between the binding of the glycosides to brush border membranes and the inhibition of  $Na^+-K^+-ATPase$ . They found that brush border membranes of rat and guinea pig bound the same amount of ouabain or digitoxin though guinea pig enzyme was 70 times more sensitive to these glycosides than rat enzyme. However, these authors have not studied the binding in the presence of  $Mg^{++}$  or ATP, thus they may be estimating only non-specific binding. It should suffice to say with these limited data that no specific binding of ouabain could be detected in rat myometrial pm.





## CHAPTER V

### CONTRACTILE EFFECTS OF CARDIAC GLYCOSIDES IN RABBIT MYOMETRIUM



## CHAPTER V

## CONTRACTILE EFFECTS OF CARDIAC GLYCOSIDES IN RABBIT MYOMETRIUM

## METHODS AND MATERIALS

## Recording of Contractions

Myometrial pieces from rabbit uterus were mounted in individual organ baths (25 ml capacity) and contractions recorded isometrically with force displacement transducers (Grass Model FT03C) connected to a Beckman RB dynograph. The temperature of the organ bath was maintained at 37°C by circulating warm water through an outer jacket with a constant temperature circulating pump. The tissues were perfused with NKR or NKR containing  $5 \times 10^{-7}$  M ouabain or  $5 \times 10^{-7}$  M digitoxin, by means of overflow to avoid exposing the tissues to air. The pieces of myometrium were allowed to equilibrate in NKR for at least 30 minutes before testing the cardiac glycoside effect. A resting tension of 0.5 g was applied to all tissues. The dynograph was calibrated with the use of weights and the sensitivity adjusted to amplify tension changes (usually 0.5 - 1 gm/cm). The chart speed was set at 5 mm/min. After equilibration a dose response curve for acetylcholine was determined and a dose of acetylcholine (Ach) which gave about a 50% response was chosen for the study. This dose of Ach was added to the tissue bath every two minutes until fairly uniform response was obtained. At this point the NKR containing cardiac glycoside was perfused and Ach was



continued to be added every two minutes. When a maximal response was obtained the perfusate was changed to NKR again and Ach addition was continued until the original Ach response returned.

A dose response curve for ouabain was plotted by using five different concentrations viz.  $10^{-8}$ ,  $5 \times 10^{-8}$ ,  $10^{-7}$ ,  $2.5 \times 10^{-7}$  and  $5 \times 10^{-7}$  Molar. For this purpose the area under the response curve was measured using a Keuffel-Esser polar planimeter (K 4656), and the increase in area compared to control response was used as a measure for the increase in tension. The results are expressed as % tension increase above control.

#### Measurement of Radioactivity

Myometrial pieces from rabbit uterus were incubated in NKR for 30 minutes at  $37^{\circ}\text{C}$  and then transferred to NKR containing  $5 \times 10^{-7}$  M  $^3\text{H}$ -ouabain. Tissues were incubated in this medium for 10 minutes. Half the tissues were then transferred to cold 0.25 M sucrose and the other half to NKR at  $37^{\circ}\text{C}$  and incubated for 10 more minutes. The tissues from  $37^{\circ}\text{C}$  NKR were transferred to cold 0.25 M sucrose. Both the batches were homogenized in cold 0.25 M sucrose and different fractions were obtained as described in Chapter 4. The plasma membrane fraction and other fractions were counted for radioactivity and their protein content measured according to methods described in earlier chapters.



## Measurement of Ion Contents

Myometrial pieces were made sodium rich by incubating in cold  $K^+$ -free Krebs Ringer overnight. These tissues were allowed to recover in  $K^+$ -free Krebs Ringer at  $37^{\circ}C$  for 20 minutes, and then transferred to NKR or NKR with different concentrations of ouabain or digitoxin. They were incubated for 10 minutes or 30 minutes. At the end of incubation the tissue wet weight was obtained after removing, blotting on filter paper and weighing in tared tubes. Tissues were then dried at  $100^{\circ}C$  in an oven for 48 hours and weighed again to get their dry weight. The Na and K contents of these tissues were determined as described in Chapter 3.





## RESULTS

### Effect of Ouabain ( $5 \times 10^{-7}$ M) on Acetylcholine Induced Contractions of Rabbit Myometrium

$5 \times 10^{-7}$  M ouabain potentiated Ach contractions and the maximal effect occurred within 10 minutes. On washing with NKR this effect disappeared in less than 10 minutes. This is shown in Figure 6.

### Effect of Digitoxin on Ach Induced Contractions of Rabbit Myometrium

$5 \times 10^{-7}$  M digitoxin had the same kind of effect as that of ouabain. This is shown in Figure 7. The potentiating effect appeared in less than 10 minutes and on washing with NKR, the effect disappeared rapidly in less than 10 minutes.

### The Dose Response of Ouabain on Rabbit Myometrium

This is shown in Figure 8.  $10^{-8}$  M and  $5 \times 10^{-8}$  M had very little potentiating effect on Ach induced contractions.  $10^{-7}$  M and  $2.5 \times 10^{-7}$  M had definite potentiating action with a mean value of  $20\% \pm 5$  and  $41\% \pm 10$  increase in tension above control tension respectively.  $5 \times 10^{-7}$  M ouabain always caused an increase in tension which was  $80\% \pm 20$  above control.



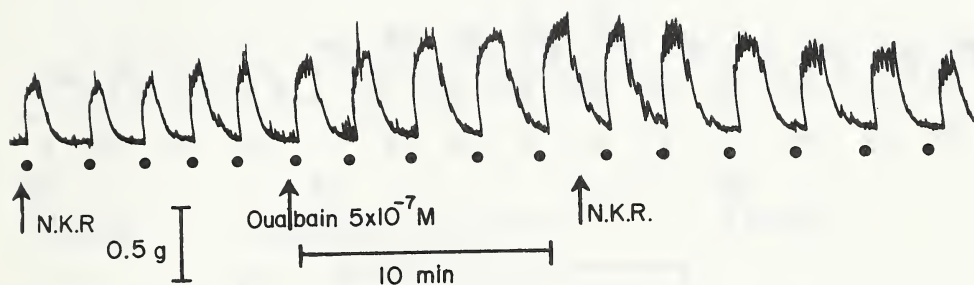


Figure 6. Effect of  $5 \times 10^{-7}$  M ouabain on acetylcholine induced contractions of rabbit myometrium.



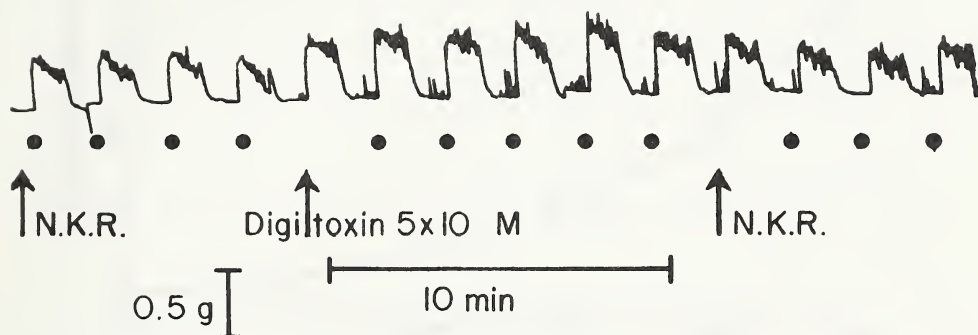


Figure 7. Effect of  $5 \times 10^{-7}$  M digitoxin on acetylcholine induced contractions of rabbit myometrium.



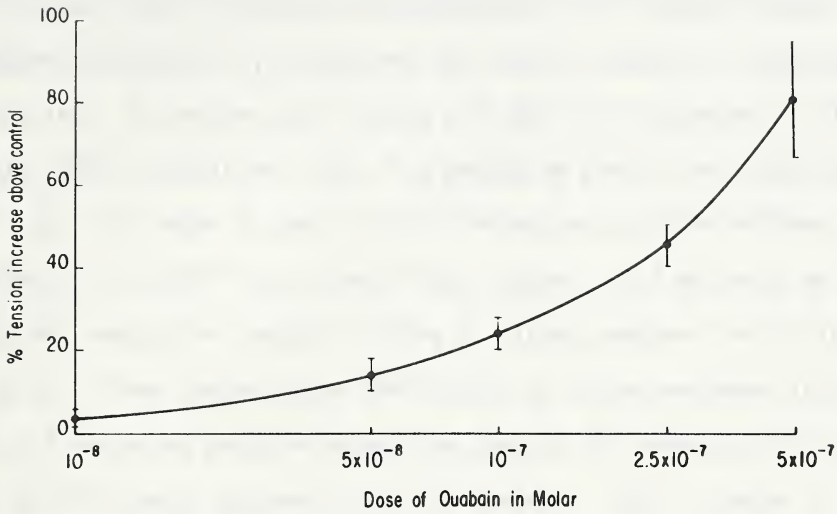


Figure 8. Dose response curve for the potentiating effect of ouabain on the acetylcholine induced contractions of rabbit myometrium.





### Binding of $5 \times 10^{-7}$ M $^3\text{H}$ -Ouabain to Plasma Membrane Fraction

Table 29 shows the subcellular distribution of  $5 \times 10^{-7}$  M  $^3\text{H}$ -ouabain after 10 minutes incubation at  $37^\circ\text{C}$  in NKR. Plasma membrane fraction ( $F_1$ ) contained the highest amount (1.7 pmoles/mg protein). On washing the tissues with NKR for 10 minutes at  $37^\circ\text{C}$  very little ouabain was lost, 1.3 pmoles/mg protein was still bound to  $F_1$ . In order to see if this binding was to  $\text{Na}^+-\text{K}^+-\text{ATPase}$ , effect of 9.2 mM  $\text{K}^+$  and 23 mM  $\text{K}^+$  was studied. 9.2 mM or 23 mM  $\text{K}^+$  did not reduce the ouabain binding to plasma membrane in 10 minutes. But in  $\text{K}^+$ -free Krebs Ringer the binding to plasma membrane increased to 3.5 pmoles/mg protein suggesting that in the absence of  $\text{K}^+$  binding to  $\text{Na}^+-\text{K}^+-\text{ATPase}$  occurred within 10 minutes. This is shown in Table 30.

### Effect of Ouabain on $\text{Na}^+$ and $\text{K}^+$ Movements of $\text{Na}^+$ -rich Tissues

Ion recovery in  $\text{Na}^+$ -rich tissues were studied to estimate inhibition of  $\text{Na}^+-\text{K}^+-\text{ATPase}$ . For these experiments paired controls were used. Table 31 shows these results.  $5 \times 10^{-7}$  M ouabain had no effect on  $\text{K}^+$  uptake when the incubation time was 10 minutes. When the recovery was in NKR the  $\text{K}^+$  content was  $70 \text{ meq} \pm 4/\text{kg}$  dry weight at the end of 10 minutes and in the presence of  $5 \times 10^{-7}$  M ouabain, it was  $68 \text{ meq} \pm 5/\text{kg}$  dry weight. The  $\text{K}^+$  content of the tissues before recovery was  $12 \text{ meq} \pm 2/\text{kg}$  dry weight. Only 19 meq could



TABLE 29

Effect of washing on  $5 \times 10^{-7}$  M  $^3\text{H}$ -ouabain binding to rabbit myometrial plasma membrane

Fractions	Control <sup>a</sup>	Washed <sup>b</sup>
F <sub>1</sub>	1.7 $\pm$ 0.30	1.3 $\pm$ 0.21
F <sub>2</sub>	0.6 $\pm$ 0.10	0.5 $\pm$ 0.09
F <sub>3</sub>	0.5 $\pm$ 0.08	0.3 $\pm$ 0.08
F <sub>4</sub>	0.3 $\pm$ 0.09	0.1 $\pm$ 0.06
PH	0.8 $\pm$ 0.10	0.6 $\pm$ 0.09
pmoles/mg protein		

<sup>a</sup> Uptake in N.K.R. with  $5 \times 10^{-7}$  M  $^3\text{H}$ -ouabain for 10 minutes at 37°C.

<sup>b</sup> Uptake in N.K.R. with  $5 \times 10^{-7}$  M  $^3\text{H}$ -ouabain for 10 minutes + washing in N.K.R. for 10 minutes at 37°C.

N = 4.



TABLE 30

Effect of  $K^+$  on the binding of  $5 \times 10^{-7}$  M  $^3H$ -ouabain to rabbit  
myometrial plasma membrane

Fractions	$K^+$ -Free	N.K.R.	9.2 mM $K^+$	23 mM $K^+$
$F_1$	$3.5 \pm 0.62$	$1.2 \pm 0.31$	$1.4 \pm 0.40$	$1.2 \pm 0.15$
$F_2$	$1.0 \pm 0.03$	$0.7 \pm 0.01$	$0.6 \pm 0.02$	$0.5 \pm 0.02$
$F_3$	$0.8 \pm 0.05$	$0.5 \pm 0.02$	$0.4 \pm 0.01$	$0.4 \pm 0.02$
$F_4$	$0.7 \pm 0.04$	$0.4 \pm 0.01$	$0.4 \pm 0.02$	$0.4 \pm 0.03$
PH	$0.9 \pm 0.03$	$0.6 \pm 0.03$	$0.5 \pm 0.04$	$0.5 \pm 0.02$
pmoles/mg protein				

N = 3.



be attributed to equilibration of potassium at 4.6 mM, with an extracellular space of 0.45 ml/g of the 56 - 58 meq/kg dry weight gain. In order to ensure that the tissues were not damaged some tissues were allowed to recover for 30 minutes in NKR and the  $K^+$  content of these tissues was  $150 \text{ meq} \pm 10/\text{kg}$  dry weight and  $Na^+$  content was reduced from  $1240 \pm 13 \text{ meq/kg}$  dry weight to  $950 \pm 8 \text{ meq/kg}$  dry weight.  $5 \times 10^{-7} \text{ M}$  ouabain inhibited the ion recovery when incubated for 30 minutes.  $K^+$  content in the presence of  $5 \times 10^{-7} \text{ M}$  ouabain for 30 minutes was  $70 \pm 8 \text{ meq/kg}$  dry weight as compared to the control value of  $128 \pm 7 \text{ meq/kg}$  dry weight.  $Na^+$  content was  $1140 \pm 14 \text{ meq/kg}$  dry weight as compared to the control value of  $980 \pm 10 \text{ meq/kg}$  dry weight.  $10^{-6} \text{ M}$  or  $10^{-5} \text{ M}$  ouabain also had very little effect on ion recovery in 10 minutes but inhibition with  $10^{-5} \text{ M}$  after 30 minutes incubation was marked. The values are given in Table 31.

#### Effect of Digitoxin on $Na^+$ - $K^+$ Movements in $Na^+$ -rich Tissues

Digitoxin, like ouabain, had no effect in 10 minutes but all the three concentrations ( $5 \times 10^{-7} \text{ M}$ ,  $10^{-6} \text{ M}$  and  $10^{-5} \text{ M}$ ) inhibited the ion recovery after 30 minutes incubation. This is shown in Table 32.





TABLE 31

Effect of ouabain on ion recovery of Na<sup>+</sup>-rich tissues

Media	10 minutes		30 minutes	
	Na <sup>+</sup> <sup>d</sup>	K <sup>+</sup> <sup>d</sup>	Na <sup>+</sup> <sup>d</sup>	K <sup>+</sup> <sup>d</sup>
K <sup>+</sup> -free <sup>a</sup>	1320 ± 12	12 ± 2	1240 ± 13	13 ± 3
N.K.R. <sup>b</sup>	1108 ± 15	69 ± 3*	950 ± 8	150 ± 10*
N.K.R.	1128 ± 13	70 ± 4	980 ± 10	128 ± 7
5 × 10 <sup>-7</sup> M ouabain <sup>c</sup>	1140 ± 20	68 ± 5	1140 ± 14	70 ± 8*
N.K.R.	1218 ± 18	69 ± 5	1018 ± 19	110 ± 8
10 <sup>-6</sup> M Ouabain	1192 ± 22	65 ± 4	1254 ± 21	72 ± 3*
N.K.R.	1315 ± 13	69 ± 6	1098 ± 11	117 ± 9
10 <sup>-5</sup> M Ouabain	1298 ± 21	61 ± 5	1318 ± 20	46 ± 2*

\* Significantly different from control (p < .05).

<sup>a</sup> Na<sup>+</sup>-rich tissues incubated in K<sup>+</sup>-free Krebs for 30 minutes at 37°C.

<sup>b</sup> Na<sup>+</sup>-rich tissues incubated in K<sup>+</sup>-free Krebs for 20 minutes and 10 minutes in N.K.R. at 37°C or 30 minutes in N.K.R. at 37°C.

<sup>c</sup> Na<sup>+</sup>-rich tissues incubated in K<sup>+</sup>-free Krebs for 20 minutes and 10 minutes in N.K.R. + ouabain or 30 minutes in the same media at 37°C.

<sup>d</sup> Na<sup>+</sup>-K<sup>+</sup> value expressed as meq of ion/kg dry weight of tissue.

N = 6.



TABLE 32

Effect of digitoxin on ion recovery of Na<sup>+</sup>-rich tissues

Media	10 minutes		30 minutes	
	Na <sup>+</sup>	K <sup>+</sup>	Na <sup>+</sup>	K <sup>+</sup>
N.K.R.	1218 $\pm$ 21	64 $\pm$ 6	1008 $\pm$ 15	100 $\pm$ 3
5 x 10 <sup>-7</sup> M Digitoxin	1209 $\pm$ 20	65 $\pm$ 4	1212 $\pm$ 14	61 $\pm$ 2*
N.K.R.	1108 $\pm$ 17	65 $\pm$ 7	958 $\pm$ 20	120 $\pm$ 10
10 <sup>-6</sup> M Digitoxin	1191 $\pm$ 20	64 $\pm$ 5	1291 $\pm$ 24	70 $\pm$ 3*
N.K.R.	1058 $\pm$ 16	69 $\pm$ 6	980 $\pm$ 18	121 $\pm$ 9
10 <sup>-5</sup> M Digitoxin	1098 $\pm$ 23	68 $\pm$ 5	1232 $\pm$ 21	52 $\pm$ 2*

\* Significantly different from control (p < .05).

N = 5.



## DISCUSSION

The dose response curve of ouabain effect on contractility suggests that the dose of  $5 \times 10^{-7}$  M was an adequate dose for myometrial tissue to potentiate acetylcholine induced contractions. The appearance of potentiating effect of ouabain and digitoxin in 10 minutes and its disappearance on washing with NKR in 10 minutes suggests that the rates of onset and offset of ouabain and digitoxin action are similar to the value in atria (Lullmann et al, 1969, Fricke and Klaus, 1971 and Thomas et al, 1970).

Lullmann and van Zweiten (1969) suggested that the inotropic effect of ouabain may be due to its inhibition of transport ATPase because they found that in red cell ghosts the ouabain inhibition of transport ATPase disappeared within 10 minutes on 10-fold dilution of ouabain-enzyme mixture. The dilution media contained 10 mM  $K^+$ . Their results are in contrast to the results of Akera et al (1971) and Allen et al (1971a) who found that  $K^+$  inhibited the dissociation of ouabain-enzyme complex. Probably these differences are due to different preparations used by these authors. However, Lullmann and van Zweiten (1969) reported that there was a parallelism between reversibility of ATPase inhibition and the disappearance of positive inotropic effect. Fricke and Klaus (1971) also found correlation between inhibition of  $Na^+-K^+-ATPase$  and inotropic action of digitalis. They showed that  $K^+$  antagonism of the glycosides studied (digitoxin,



K-strophanthidin and strophanthidin-Bromo acetate SBA) and also the reversibility of inhibition of  $\text{Na}^+-\text{K}^+$ -ATPase was closely related to inotropic effect. However, with SBA they found 30% irreversible inhibition of ATPase but complete reversal of inotropic effect (see Chapter 2). There are other reports (Schwartz et al, 1969, Akera et al, 1969, Prindle et al, 1970) supporting the involvement of transport ATPase in inotropic effect of digitalis. However, most of these studies have not correlated the binding of cardiac glycosides to  $\text{Na}^+-\text{K}^+$ -ATPase to contractile effect. When such studies were done the time course of uptake and contractile effect were not similar, e.g., Prindle et al (1970) correlated the binding to microsomal fraction with contractile effect. The contractile effect appeared within 20 minutes but they studied the uptake only after 45 minutes incubation of tissues with  $^3\text{H}$ -digoxin (see Chapter 2). Thus these results are not conclusive.

Results of Thomas et al (1970) and Roth-Schechter et al (1970) provide evidence against the involvement of  $\text{Na}^+-\text{K}^+$ -ATPase in inotropic action of cardiac glycosides. They found that SBA, an irreversible inhibitor of  $\text{Na}^+-\text{K}^+$ -ATPase, had the same kind of kinetic behaviour with respect to contractile effect as that of ouabain, a reversible inhibitor of  $\text{Na}^+-\text{K}^+$ -ATPase. The contractile effect of SBA was completely reversible. Present results also show a dissociation between binding of ouabain to  $\text{Na}^+-\text{K}^+$ -ATPase inhibition and contractile effect. The dissociation between binding and contractile effect was





shown in two ways. It was first shown that the binding to plasma membrane fraction was not washed off completely in 10 minutes whereas the contractile effect was washed off completely in 10 minutes. Allen et al (1971) and Akera and Brody (1971) have shown that  $K^+$  stabilizes the  $Na^+-K^+-ATPase$ -ouabain complex. As in the present studies NKR was used for contractile and washing experiments, it can be argued that  $K^+$  in NKR might have prevented the dissociation of ouabain. This would only support the suggestion that binding to  $Na^+-K^+-ATPase$  can be dissociated from contractile effect because the same medium reversed the contractile effect completely. Tobin and Sen (1970) on the other hand, found a complete dissociation of ouabain-enzyme complex at  $37^{\circ}C$  in 15 minutes. Thus it was of interest to see if the ouabain binding to plasma membrane was in fact related to  $Na^+-K^+-ATPase$ . Therefore the effect of high  $K^+$  on this binding was studied. 9.2 mM  $K^+$  or 23 mM  $K^+$  did not reduce the binding of  $5 \times 10^{-7}$  M  $^3H$ -ouabain to plasma membrane fraction (PM) in 10 minutes. But the binding increased in  $K^+$ -free media. These results can be explained on the basis of rate of ouabain binding to PM in the presence and absence of  $K^+$ . It is well known that ouabain binds rapidly to  $Na^+-K^+-ATPase$  in the absence of  $K^+$  (Albers et al, 1968, Matsui and Schwartz, 1967, and Barnett, 1970) and  $K^+$  reduces this rate of binding. Thus in  $K^+$ -free Krebs Ringer the rate is much faster than the binding in NKR as high  $K^+$  Ringer. Therefore in  $K^+$ -free Ringer there is an increase in binding of ouabain to PM in



10 minutes and this may be related to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  dependent binding. In NKR, probably very little binding to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  occurs in 10 minutes and therefore 9.2 mM  $\text{K}^+$  or 23 mM  $\text{K}^+$  had no further reducing effect. These results suggest that most of the binding in 10 minutes occurs at non-specific, i.e., not related to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  under the conditions where contractile effect is studied. These results, thus, substantiate the suggestion that there is a dissociation between contractile effect of ouabain and its binding to  $\text{Na}^+\text{-K}^+\text{-ATPase}$ .

The results on the effect of ouabain on the  $\text{Na}^+$  and  $\text{K}^+$  movements of  $\text{Na}^+$ -rich tissues suggested that no inhibition of the  $\text{Na}^+$  pump occurred in 10 minutes with  $5 \times 10^{-7}$  M ouabain.  $\text{Na}^+$ -rich tissues were used for the reasons given by Carey et al (1959) and Rangachari (1971).

These reasons are:

1. The changes in total  $\text{Na}^+$  content and  $\text{K}^+$  content can be taken as reasonable indices for active cation transport, since the extrusion of  $\text{Na}^+$  is "far beyond the small amount that could be attributed to diffusion from inter-spaces to equilibrate with the external fluid" (Carey et al, 1959).
2. In the absence of external  $\text{K}^+$ , the  $\text{Na}^+$  pump is inhibited. The addition of sufficient  $\text{K}^+$  would be expected to activate the  $\text{Na}^+$  pump at a maximal rate in  $\text{Na}^+$ -rich tissues and the effect of ouabain or digitoxin would be more pronounced under such conditions (Rangachari, 1971).



Rangachari (1971) showed that Na-rich myometrium of rat recovered partially within 15 minutes at 37°C in NKR. Similar results were obtained in the present study. There was considerable recovery in 10 minutes and 30 minute incubation enhanced this recovery. Ouabain when present in the concentration of  $5 \times 10^{-7}$  M did not affect recovery in 10 minutes. Even  $10^{-5}$  M ouabain did not inhibit the recovery in 10 minutes. However, on 30 minutes exposure to  $5 \times 10^{-7}$  M ouabain, the  $K^+$  uptake was inhibited. But as the contractile effect had reached its peak in 10 minutes while the ion recovery was not inhibited in 10 minutes, a clear dissociation between contractile effect and inhibition of  $Na^+$  pump has been shown. Similar results were obtained using digitoxin as well. These results are in disagreement with the suggestion of Lee et al, Langer (1971) and Glynn (1969) that the cause of increased contractility by cardiac glycosides is the inhibition of  $Na^+-K^+-ATPase$  which would cause an increase in the intracellular  $Na^+$  which in turn releases  $Ca^{++}$  to cause an increased contraction. These results also disagree with the results of Besch et al, Allen et al, and Schwartz et al, who related  $Na^+-K^+-ATPase$  inhibition to contractile effect. However, the results support the results of Thomas et al (1970) and Roth-Schechter et al, (1970) and suggest that  $Na^+-ATPase$  inhibition may not be necessary for contractile effect. These results also suggest that there may be two receptor sites for the cardiac glycosides on the membrane, one involved with the inotropic effect and the other involved with  $Na^+-K^+-ATPase$



inhibition. The case of disappearance of contractile effect suggests an easily reversible interaction between the receptor and cardiac glycosides. There was about 0.4 pmoles of ouabain released from the PM fraction during 10 minutes washout. This may have been responsible for contractile effect. Recently, Kim et al (1972) have shown a correlation of the inotropic effect with the loosely bound digoxin to the microsomal fraction. They suggest that the loose binding of digoxin to microsomal fraction is responsible for positive inotropic effect. It is not, however, possible to speculate from these results as to how this binding brings about increase in contractility.







## CONCLUSION



## CONCLUSION

In Chapter I, I said that this thesis would attempt to answer four major questions. I shall now recapitulate these questions and some of the answers obtained from the present study.

1. Is there a correlation between lipid solubility and uptake of cardiac glycosides?

The uptake of cardiac glycosides by rabbit myometrium showed that T/M ratio was probably dependent on lipid solubility. Digitoxin which is lipophilic\* had a higher T/M (3.0 ml/g) than ouabain (0.6 ml/g) which has a lower lipid solubility.

2. Do these glycosides bind to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  to the same extent with similar characteristics?

Though the T/M (1.3 ml/g) for ouabain in  $\text{K}^+$ -free media was lower than T/M (3.0 ml/g) for digitoxin, more of ouabain was probably bound to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  than digitoxin in  $\text{K}^+$ -free media. Binding of both the glycosides was  $\text{Na}^+$ -dependent, but 9.2 mM  $\text{K}^+$  did not reduce the binding of digitoxin as it did that of ouabain. This suggests that these two glycosides may be binding to different forms of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . In binding to isolated plasma membrane experiments, when equal concentration of ouabain and digitoxin ( $5 \times 10^{-8}$  M) were used in the presence of  $\text{Mg}^{++}$ ,  $\text{Na}^+$  and ATP (and washed twice with cold distilled water), more ouabain was bound (2.7 pmoles/mg protein) as compared to digitoxin binding (0.9 pmoles/mg protein) while 5 mM  $\text{K}^+$  reduced ouabain binding,

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\* Partition coefficients of digitoxin and ouabain in chloroform/water system are 89.7 and 0 respectively (Greenberger et al, 1969).



even 20 mM  $K^+$  had only a slight effect on digitoxin binding. Therefore, polar glycosides seem to have a higher affinity for the  $E_2 - P$  form of  $Na^+ - K^+ - ATPase$  than non-polar glycosides. Before washing equal amounts of digitoxin and ouabain were bound to  $F_1$  (about 3.2 pmoles) but after two washings 70% of digitoxin was released while only 23% of ouabain was released. Thus most of the ouabain binding was specific while digitoxin binding was non-specific. It is also possible that these two glycosides bind to conformationally different forms of  $Na^+ - K^+ - ATPase$ . In spite of their similar contractile effects, they bind differently to  $Na^+ - K^+ - ATPase$  which has been suggested to be the receptor for inotropic effect (Besch et al, 1970 and Akera et al, 1970).

### 3. What is the difference in binding characteristics of cardiac glycosides between sensitive and insensitive species?

Ouabain binding to rabbit myometrial pm (sensitive) was  $Na^+ - K^+ - ATPase$  dependent whereas such a dependence could not be shown in the case of rat myometrium (insensitive). Some of the reasons for the failure to see this dependence could have been a higher non-specific uptake unrelated to  $Na^+ - K^+ - ATPase$  or an extremely labile specific binding. Though the control experiments of binding to subcellular fractions showed that no exchange of  $^3H$ -ouabain occurred during isolation of pm, it is still possible that the binding was labile and released during homogenization. This could go undetected if no reuptake occurred. Experiments on binding to isolated PM also failed to



show any specific binding with and without washing (see Results of Chapter IV).

4. Is there a correlation between binding of cardiac glycosides to  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and contractile effect?

Present study showed that there were instances of lack of correlation between contractile effect and binding to and inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  by cardiac glycosides. These two effects could be dissociated.

It is evident that these answers raise several problems which need further investigation. The following are some of them:

(a) To prove whether digitoxin bound to  $\text{E}_1$  - P form of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  or not in the intact tissue, it should be tested whether the binding was ATP-dependent. This requires the use of metabolic inhibitors to deplete the tissue of ATP. It is also necessary to prove that these inhibitors were not acting on  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . Therefore, an experiment using the metabolic inhibitors discretely (see Rangachari, 1971) and supplying substrates to circumvent this inhibition is suggested so that both inhibition and recovery of binding of digitoxin could be studied.

(b) The absence of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ -dependent binding in rat myometrium is difficult to explain. Because ouabain inhibits the enzyme partially purified from rat myometrium and  $\text{K}^+$  antagonism of this inhibition has also been shown by Kidwai et al (unpublished experiments). Possibly such a binding could be demonstrated with a







highly purified  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . Although we are aware of the extreme difficulty of purifying an enzyme, it would seem that such a purified preparation will have to be obtained before this problem can be solved.

(c) If  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is not the receptor for inotropic effect of cardiac glycosides, then how do these drugs act to bring about inotropic effect? In Chapter V evidence was presented that about 0.4 pmoles/mg protein of ouabain bound to PM appeared to be associated with contractile effect. It would be worthwhile to see if these glycosides have any effect on  $\text{Ca}^{++}$  binding to PM. These drugs might release  $\text{Ca}^{++}$  from PM by some other mechanism rather than the inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . It is also possible that binding to endoplasmic reticulum fraction ( $F_2$ ) was responsible for the contractile effect (though much less contractile dependent binding occurred to  $F_2$  than to  $F_1$ ). Further studies are needed to confirm this possibility. Lee et al (1969) have shown that cardiac glycosides alter the  $\text{Ca}^{++}$  binding property of SR and this effect may involve binding of cardiac glycosides to SR. The contractile dependent binding of ouabain was easily reversible and as the washout time of contractile effect corresponds with the washing off time of the drug from extracellular space (less than 10 minutes), these glycosides must act on such cellular organelle which is easily accessible from extracellular space. There have been suggestions (Kulczycky and Mainwood, 1972) that SR is open to extracellular space. If this suggestion is right then SR may be the site of action of cardiac glycosides.



(d) There are very few studies which have compared the inhibitory effects of ouabain and digitoxin using similar  $\text{Na}^+\text{-K}^+\text{-ATPase}$  preparations. Such studies may throw some light on the mechanism of inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  by these two glycosides which have different binding properties.



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The first thing I noticed when I stepped out of the car was the  
 warm, humid air. It felt like a giant hand reaching out to  
 embrace me. I took a deep breath, savoring the scent of  
 tropical flowers and the salty breeze from the ocean.  
 The sun was shining brightly, casting a golden glow over  
 everything. I could see the palm trees swaying gently in the  
 breeze, their fronds creating a rhythmic pattern against the  
 sky. The sound of the waves crashing against the shore was  
 soothing, a melody that had been playing in my mind for  
 years. I felt a sense of peace and tranquility that I had never  
 experienced before. It was as if I had found a hidden world  
 where time stood still, and all my worries and problems  
 were left behind. I smiled, feeling a sense of joy and  
 wonder. This was my chance to escape the mundane and  
 embrace the beauty of nature. I took another deep breath,  
 letting the sun and the sea fill my lungs. I felt like I was  
 reborn, with a fresh start and a new beginning. The world  
 around me was so beautiful, so vibrant, and so full of life.  
 I knew that this was the place I needed to be. I was  
 finally free, and I was going to make the most of it. I  
 turned around, looking back at the car with a sense of  
 accomplishment. I had done it. I had found my paradise.  
 I took one last look at the ocean, feeling a sense of  
 awe and wonder. The waves were so powerful, so majestic,  
 and so beautiful. I knew that I was going to love every  
 minute of this. I turned back around, ready to embrace  
 the world that was waiting for me. I felt like I was  
 stepping into a new chapter of my life, one that was  
 full of adventure, discovery, and joy. I was going to  
 make the most of this, and I was going to love every  
 minute of it.

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The first part of the book is devoted to a general introduction to the subject of the history of the English language, and to a discussion of the various factors which have influenced its development.

The second part of the book is devoted to a detailed study of the history of the English language from the time of its first appearance in the world to the present day.

The third part of the book is devoted to a study of the various dialects of the English language, and to a discussion of the factors which have influenced their development.

The fourth part of the book is devoted to a study of the various literary forms of the English language, and to a discussion of the factors which have influenced their development.

The fifth part of the book is devoted to a study of the various grammatical forms of the English language, and to a discussion of the factors which have influenced their development.

The sixth part of the book is devoted to a study of the various phonetic forms of the English language, and to a discussion of the factors which have influenced their development.

The seventh part of the book is devoted to a study of the various orthographic forms of the English language, and to a discussion of the factors which have influenced their development.

The eighth part of the book is devoted to a study of the various lexicographical forms of the English language, and to a discussion of the factors which have influenced their development.

The ninth part of the book is devoted to a study of the various etymological forms of the English language, and to a discussion of the factors which have influenced their development.



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1. The first part of the paper is devoted to the study of the properties of the function  $f(x)$  defined by the equation

$$f(x) = \frac{1}{2} (f(x-1) + f(x+1))$$

where  $f(x)$  is a function defined on the interval  $[0, 1]$  and satisfying the conditions  $f(0) = 0$  and  $f(1) = 1$ . It is shown that the function  $f(x)$  is uniquely determined by these conditions and that it is a linear function.

2. In the second part of the paper

the author considers the problem of finding the maximum value of the function  $f(x)$  on the interval  $[0, 1]$  under the condition that  $f(x)$  is a function satisfying the equation

$$f(x) = \frac{1}{2} (f(x-1) + f(x+1))$$

and the conditions  $f(0) = 0$  and  $f(1) = 1$ . It is shown that the maximum value of the function  $f(x)$  is equal to  $\frac{1}{2}$ .

3. In the third part of the paper

the author considers the problem of finding the minimum value of the function  $f(x)$  on the interval  $[0, 1]$  under the condition that  $f(x)$  is a function satisfying the equation

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5. In the fifth part of the paper

Wilbrandt, W. (1966). Mechanism of action of cardiac glycosides.

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the following conditions are satisfied: (i)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra;

(ii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (iii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra;

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(xxiii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxiv)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxv)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra;

(xxvi)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxvii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxviii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra;

(xxix)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxx)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxxi)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra;

(xxxii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxxiii)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra; (xxxiv)  $\mathcal{A}$  is a  $\mathcal{P}$ -algebra;













**B30042**